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(71) Applicant *(for all designated States except US)*: **JOHNS HOPKINS UNIVERSITY** [US/US]; 720 Rutland Avenue, Baltimore, MD 21205 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **WU, Tzyy-Choou** [US/US]; 11002 Nacirema Lane, Stevenson, MD 21153 (US). **HUNG, Chien-Fu** [US/US]; 6605 Sanzo Road, Baltimore, MD 21209 (US).

(74) Agent: **LIVNAT, Shmuel**; Venable, Baetjer, Howard & Civiletti, LLP, P.O. Box 34385, 1201 New York Avenue, NW, Suite 1000, Washington, DC 20043-9998 (US).

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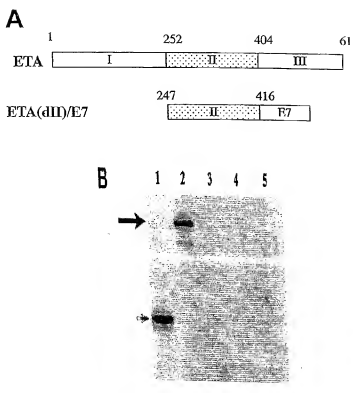
(54) Title: SUPERIOR MOLECULAR VACCINE LINKING THE TRANSLOCATION DOMAIN OF A BACTERIAL TOXIN TO AN ANTIGEN



(57) Abstract: Nucleic acids encoding a chimeric or fusion polypeptide which polypeptide comprises a first domain comprising a translocation polypeptide; and a second domain comprising at least one antigenic peptide are disclosed. The preferred translocation polypeptide is a bacterial toxin translocation polypeptide, such as domain II of *Pseudomonas aeruginosa* exotoxin A (ETA(dII)). Such nucleic acids, expression vectors thereof, and cells expressing these vectors are used as immunogenic or vaccine compositions in a method for enhancing an antigen specific immune response, a method of increasing the numbers of $CD8^+$ CTLs specific for a selected desired antigen in a subject, or a method of inhibiting the growth of a tumor in a subject.

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SUPERIOR MOLECULAR VACCINE LINKING THE TRANSLOCATION DOMAIN OF A BACTERIAL TOXIN TO AN ANTIGEN

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention in the fields of molecular biology, immunology and medicine relates to a chimeric nucleic acid, preferably DNA, encoding a fusion protein and its use as a vaccine to enhance immune responses, primarily cytotoxic T lymphocyte (CTL) responses to specific antigens such as tumor or viral antigens. The fusion protein comprises an antigenic polypeptide fused to a bacterial toxin translocation protein that promotes processing via the MHC class I pathway and selective induction of immunity mediated by $CD8^+$ antigen-specific CTL.

Description of the Background Art

Cytotoxic T lymphocytes (CTL) are critical effectors of antitumor responses (reviewed in Refs 1-3). Activated CTL are effector cells that mediate antitumor immunity by direct lysis of their target tumor cells or by releasing of cytokines that orchestrate immune and inflammatory responses that interfere with tumor growth or metastasis. Depletion of $CD8^+$ CTL leads to the loss of antitumor effects of several cancer vaccines (4, 5). Therefore, the enhancement of antigen presentation through the MHC class I pathway to $CD8^+$ T cells has been a primary focus of cancer immunotherapy.

Naked DNA vaccines have emerged recently as attractive approaches for vaccine development (reviewed in 6-11). DNA vaccines generated long-term cell-mediated immunity (reviewed in 12). In addition, DNA vaccines can generate $CD8^+$ T cell responses in vaccinated humans (13). However, one limitation of these vaccines is their lack of potency, since the DNA vaccine vectors generally do not have the intrinsic ability to be amplified and to spread *in vivo* as do some replicating viral vaccine vectors. Furthermore, some tumor antigens such as human papillomavirus-16 (HPV-16) E7 (5) are weak immunogens. Therefore, there is a need in the art for strategies to enhance DNA vaccine potency, particularly for more effective cancer immunotherapy.

The present inventors and their colleagues recently demonstrated that linkage of HPV-16 E7 antigen to *Mtb* heat shock protein 70 (Hsp70) leads to the enhancement of DNA vaccine potency (5). (See also USSN 09/501,097, filed 09 February 2000; and USSN 099/421,608, filed 20 October 1999, from which the present application claims priority) Immunization with HSP complexes isolated from tumor or virus-infected cells induced potent anti-tumor immunity (Janetzki, S *et al.*, 1998. *J Immunother 21*:269-76) or antiviral immunity (Heikema, AE *et al., Immunol Lett* 57:69-74). In addition, immunogenic HSP-peptide complexes could be reconstituted *in vitro* by mixing the peptides with HSPs (Ciupitu, AM *et al.*, 1998. *J Exp Med* 187:685-91). Furthermore, HSP-based protein vaccines have been created by fusing antigens to HSPs (Suzue, K *et al.*, 1996. *J Immunol* 156:873-9). The results of these investigations point to HSPs a attractive candidates for use in immunotherapy. However, prior to the present inventors' work, HSP vaccines were all peptide/protein-based vaccines or, in more recent cases, were in the form of naked DNA. To date, there have been no reports of HSPs incorporated into self-replicating RNA vaccines.

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SUMMARY OF THE INVENTION

The growing understanding of the antigen presentation pathway creates the potential for designing novel strategies to enhance vaccine potency. One strategy taken by the present inventors in the present invention to enhance the presentation of antigen through the MHC class I pathway to $CD8^+$ T cells is the exploitation of the translocation features of certain bacterial toxins such as *Pseudomonas aeruginosa* exotoxin A (ETA) (reviewed in Goletz, TJ *et al.*, Hum Immunol. *54:* 129-136, 1997). ETA is one of several secreted bacterial toxins that can covalently modify particular proteins in mammalian cells through the toxin's translocation.

Molecular characterization of ETA has revealed three functional domains (Hwang, J *et al.*, Cell. *48:* 129-136, 1987). Domain I is responsible for binding to a cell surface receptor (Guidi-Rontani, *et al.*, Mol Microbiol. *1:* 67-72, 1987). Domain II is responsible for translocation to the cytosol (Jinno, Y *et al.*, J Biol Chem. *264:* 15953-15959, 1989; Siegall, CB *et al.*, Biochemistry. *30:* 7154-7159, 1991; Prior, TI *et al.*, Biochemistry. *31:* 3555-3559, 1992). Domain III is responsible for the toxic activity by binding to ADP-ribosyl transferase (Chaudhary, VK *et al.*, Proc Natl Acad Sci U S A. *87:* 308-312, 1990). In particular, domain II (dII) of ETA (abbreviated ETA(dII)) has been used to engineer a chimeric multidomain protein to deliver DNA into the cytosol (Fominaya, J *et al.*, J Biol Chem. *271:* 10560-10568, 1996; Fominaya, J *et al.*, Gene Ther. *5:* 521-530, 1998). This capacity to facilitate translocation from extracellular and vesicular compartments into the cytoplasm represents an opportunity to enhance class I presentation of exogenous antigen to $CD8^+$ T cells.

The present inventors created a novel fusion of the translocation domain (domain II) of *Pseudomonas aeruginosa* exotoxin A (ETA(dII)) with a model tumor antigen, human papillomavirus type 16 (HPV-16) E7, in the context of a DNA vaccine. In *in vitro* studies, the inventors showed that cells transfected with ETA(dII)/E7 DNA or dendritic cells (DCs) pulsed with lysates containing ETA(dII)/E7 protein exhibited enhanced major histocompatibility (MHC) class I presentation of E7 antigen. Vaccination of mice with ETA(dII)/E7 DNA generated a dramatic increase in the number of E7-specific $CD8^+$ T cell precursors (approximately 30-fold compared to wild-type E7 DNA) and converted a less effective DNA vaccine into one with significant potency against HPV-16 E7-expressing murine tumors via a CD8-dependent pathway. These results indicate that fusion of the translocation domain of a bacterial toxin to an antigen may greatly enhance vaccine potency.

Thus the present invention is directed to a nucleic acid encoding a chimeric or fusion polypeptide which polypeptide comprises:

(a) a first domain comprising a translocation polypeptide; and

(b) a second domain comprising an antigen which comprises at least one antigenic peptide.

In the above nucleic acid, the translocation polypeptide is preferably a bacterial toxin translocation polypeptide, more preferably ETA(dII).

The above nucleic acid is preferably SEQ ID NO:3 or a homologue thereof.

The above nucleic preferably comprises a nucleotide sequence that encodes a translocation polypeptide which sequence is included in SEQ ID NO:1.

In the above nucleic acids, the antigenic peptide preferably comprises an epitope that binds to, and is presented on the cell surface by, MHC class I proteins. The epitope is preferably between about 8 and about 11 amino acid residues in length. The "antigen" encoded by the nucleic acid may consist of multiple epitopes that occur together naturally in a single protein or which originate from different proteins and are artificailly fused in this construct to create a multi-epitope or multi-antigen domain.

Preferably the above antigen is one which (i) is derived from a pathogen, including a pathogenic mammalian cell, a a microorganism or a virus; or (ii) cross-reacts with an antigen of the pathogen. The above virus may be a human papilloma virus; the antigen is preferably the HPV-16 E7 or E6 protein or an epitope thereof. In one embodiment, the E7 polypeptide is a nononcogenic form. The pathogen may also be a bacterium. A preferred pathogenic mammalian cell is a tumor cell or cancer cell, and the antigen is a tumor-specific or tumor-associated antigen.

The above nucleic acid may be operatively linked to a promoter, preferably one which is expressed in an antigen presenting cell (APC), more preferably in a dendritic cell.

The present invention also provides an expression vector comprising any of the above nucleic acid molecules, operatively linked to a promoter and, optionally, to one or more regulatory elements that enhance expression of the nucleic acid in a cell.

The above expression vector may be a viral vector or a plasmid, including a self-replicating RNA replicon.

In the above expression vector, the translocation polypeptide is preferably ETA(dII).

Also provided is a particle comprising the above nucleic acid or expression vector. The particle preferably comprises a material, such as gold, that is suitable for introduction into a cell or an animals by particle bombardment.

The present invention is also directed to a cell which has been modified to comprise the above nucleic acid or the above the expression vector, and which cell expresses the nucleic acid. Preferably, the cell is an APC, such as a dendritic cell, a keratinocyte, a macrophage, a monocyte, a B lymphocyte, a microglial cell, an astrocyte, or an activated endothelial cell.

Also provided is a chimeric polypeptide comprising

(a) a first domain comprising a translocation polypeptide; and

(b) a second domain comprising an antigen which comprises at least one antigenic peptide.

The translocation polypeptide is preferably a bacterial toxin translocation polypeptide, more preferably, ETA(dII). Preferably, the translocation polypeptide comprises SEQ ID NO:3 or a homologue thereof.

The above chimeric polypeptide is preferably encoded by a nucleic acid as described above.

Preferably, in the chimeric polypeptide, the antigenic peptide comprises an epitope that binds to, and is presented on the cell surface by, MHC class I proteins.

In the above chimeric peptide, the translocation domain and the antigenic peptide(s) may be linked by a chemical linker.

Preferably, the chimeric polypeptide above is a fusion polypeptide.

The first domain may be either N-terminal or C-terminal to the second domain.

The present invention is also directed to a pharmaceutical composition capable of inducing or enhancing an antigen specific immune response, comprising a pharmaceutically acceptable carrier or excipient and any one or more of: (a) the above nucleic acid; (b) the above expression vector; (c) the above particle; (d) the above cell; or (e) the above chimeric polypeptide.

In another embodiment, the invention is directed to a method of enhancing an antigen specific immune response comprising administering an effective amount of a composition comprising: (a) the above nucleic acid; (b) the above expression vector; (c) the above particle; (d) the above cell; or (e) the above chimeric polypeptide, thereby inducing or enhancing the antigen specific immune response.

In the above method, the antigen specific immune response is preferably mediated at least in part by $CD8^+$ cytotoxic T lymphocytes (CTL).

In the above methods, the composition may be administered *ex vivo*, for example, o APCs, preferably human APCs, such as ones from a live subject. Preferred APCs are DCs. This method may further comprise administering the *ex vivo*-treated APCs to a histocompatible subject.

In another embodiment of the above methods, the composition is administered *in vivo*, preferably to a human. Preferred routes of administration are intramuscularly, intradermally, or

subcutaneously. In administering the composition to a subject with a tumor, the route may be intratumoral or peritumoral.

Also provided is a method of increasing the numbers of $CD8^+$ CTLs specific for a selected desired antigen in a subject comprising administering an effective amount of a composition comprising: (a) the above nucleic acid; (b) the above expression vector; (c) the above particle; (d) the above cell; or (e) the above chimeric polypeptide, wherein the antigenic peptide comprises an epitope that binds to and is presented on the cell surface by MHC class I proteins, thereby increasing the numbers of antigen-specific $CD8^+$ CTLs.

In another embodiment, the invention provides a method of inhibiting the growth of a tumor in a subject comprising administering an effective amount of a composition comprising: (a) the above nucleic acid; (b) the above expression vector; (c) the above particle; (d) the above cell; or (e) the above chimeric polypeptide, thereby inhibiting growth of the tumor. In this method the administering may be intratumoral or peritumoral.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A and 1B. Chimeric ETA(dII)/E7 DNA construct and characterization of E7 protein expression. Fig. 1A is a schematic diagram showing the constructs of full-length ETA and the chimeric ETA(dII)/E7 gene. The DNA fragment encoding ETA(dII) (aa 247-416) is depicted in the spotted box. The fragment encoding HPV-16 E7 (aa 1-96) is depicted in the white box. Fig. 1B shows a Western blot analysis characterizing the expression of E7/GFP protein in cells transfected with E7/GFP or ETA(dII)/E7/GFP DNA. *Lane* 1, lysates from cells transfected with E7/GFP DNA; *Lane* 2, lysates from cells transfected with ETA(dII)/E7/GFP DNA; *Lane* 3, concentrated culture medium from cells transfected with E7/GFP DNA; *Lane* 4, concentrated culture medium from cells transfected with ETA(dII)/E7/GFP DNA; *Lane* 5, lysates from nontransfected 293 D^bK^b cells as a negative control. Lysates from E7/GFP DNA-transfected 293 D^bK^b cells revealed a protein band with a size of approximately Mr 30,000 corresponding to E7/GFP protein in *Lane 1,* as indicated by the *short arrow*). Lysates from ETA(dII)/E7/GFP DNA-transfected 293 D^bK^b cells generated a protein band with a M_r of approximately 56 kDa corresponding to ETA(dII)/E7/GFP protein in *Lane 2,* as indicated by the *long arrow*. E7/GFP DNA-transfected cells exhibited levels of protein expression comparable with that of ETA(dII)/ E7/GFP DNA-transfected cells.

Figure 2A and 2B show results of CTL assays. Fig. 2A demonstrates enhanced presentation of E7 through the MHC class I pathway of cells transfected with ETA(dII)/E7 DNA. 293 D^bK^b cells transfected with various DNA constructs served as target cells. Various E/T ratios were used, with D^b-restricted E7-specific $CD8^+$ effector T cells. Fig. 2B demonstrates enhanced MHC class I presentation of E7 in bone marrow-derived DCs pulsed with cell lysates containing chimeric ETA(dII)/E7 protein. Bone marrow-derived DCs were pulsed with cell lysates from various DNA-transfected 293 D^bK^b cells at different concentrations as described in Example I. These assays were performed at a fixed E:T ratio (9/1) using D^b-restricted E7-specific $CD8^+$ effector T cells.

Figure 3A and 3B show results of intracellular cytokine staining and flow cytometric analysis. In the results shown in Fig. 3A, the number of IFN-γ-producing E7-specific $CD8^+$ T cells was determined using flow cytometry in the presence of MHC class I restricted E7 peptide (aa 49-57). (Fig. 3B) The number of IFN-γ-producing E7-specific $CD4^+$ T cells was determined using flow cytometry in the presence MHC class II restricted E7 peptide (aa 30-67). Results are from one representative experiment of two performed.

Figure 4A, 4B and 4C shows results of *in vivo* tumor protection and therapy experiments using the TC-1 tumor, and demonstrate the role of lymphocyte subsets. Fig. 4A shows results of an *in vivo* tumor protection experiment. 100% of mice receiving ETA(dII)/E7 DNA vaccination remained tumor-free 60 days after TC-1 challenge. Fig. 4B shows results of an *in vivo* tumor therapy experiment. The ETA(dII)/E7 group had the fewest pulmonary nodules compared to the other vaccinated groups (one-way ANOVA, $p<0.001$). Results are expressed as mean number of lung nodules ± SEM. Fig. 4C shows results of *in vivo* antibody depletion experiments to determine which lymphocyte subsets are responsible for the tumor protection of the ETA(dII)/E7 DNA composition. Depletion of $CD4^+$, $CD8^+$ and $NK1.1^+$ cells was initiated one week prior to tumor challenge and continued for 63 days after tumor challenge.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The ability of the ETA(dII) polypeptide to facilitate translocation from the endosomal/lysosomal compartments to the cytoplasm suggested to the present inventors that at it may lead to the enhancement of MHC class I presentation of exogenous antigen if physically linked to the antigen. They therefore engineered an immunogenic DNA composition, or DNA

vaccine, encoding ETA(dII) linked to a model antigen, which was predicted to enhance MHC class I presentation of this antigen to $CD8^+$ T cells and thereby enhance vaccine potency. The model antigen for vaccine development was the E7 protein of the human papilloma virus HPV-16 E7. E7 is important in the induction and maintenance of cellular transformation by the virus and is co-expressed in most HPV-containing cervical cancers as well as their precursor lesions (Wu, TC, Curr Opin Immunol. *6:* 746-754, 1994). Therefore, vaccines targeting E7 are useful for preventing and/or and treating HPV-associated cervical malignancies.

The results presented herein indicate that vaccination with a chimeric ETA(dII)/E7 DNA vaccine enhanced MHC class I presentation of E7, leading to a dramatic increase in the number of E7-specific $CD8^+$ T cell precursors. Furthermore, the ETA(dII)/E7 DNA vaccine generated potent antitumor effects against subcutaneous E7-expressing tumors and already established E7-expressing metastatic lung tumors. These results indicate that fusion of the translocation domain of ETA to an antigen greatly enhances MHC class I presentation of the antigen. This represents a novel strategy to improve vaccine potency.

The invention provides compositions and methods for enhancing the immune responses, particularly cytotoxic T cell immune responses, induced by *ex vivo* or *in vivo* administration of chimeric polypeptides or, preferably, nucleic acid vaccines that encode these chimeric polypeptides. The preferred chimeric or fusion polypeptide comprises(1) at least one first polypeptide or peptide that, upon introduction to cells of the host immune system, *in vitro* or *in vivo*, promotes (a) processing via the MHC class I pathway and/or (b) development or activity of APCs, primarily DCs, and (2) at least one second polypeptide or peptide that is an antigenic polypeptide or peptide in the host.

As noted, in a preferred embodiment, the chimeric or fusion polypeptides are "indirectly" administered by administration of a nucleic acid that encodes the chimeric molecule; the nucleic acid construct, and thus the fusion protein, is expressed *in vivo*. The chimeric nucleic acids are administered in the form of DNA vaccines, either naked DNA or suicidal DNA, or a self-replicating RNA replicons.

The fusion protein comprises at least two domains or repeats thereof. A preferred embodiment of the first type of domain is a polypeptide that facilitates translocation from the endosomal/lysosomal compartments to the cytoplasm, thereby promoting processing via the MHC class I pathway. The most preferred polypeptide is ETA(dII). Other useful translocation

polypeptides may be similar pathogenic bacterial toxins from *Diptheria*, *Clostridium*, *Botulinum*, *Bacillus*, *Yersinia*, *Vibrio cholerae*, or *Bordetella pertussis*, or active fragments or domains of any of the foregoing polypeptides.

The second "domain" comprises a peptide or polypeptide, that includes one or several epitopes, derived from an antigen against which an immune response is desired, preferably a tumor antigen. In a preferred embodiment, the peptide comprises at least one MHC class I-binding peptide epitope that helps stimulate CD8+ CTLs and is recognized by such cells and their precursors.

The order in which the two (or more) component polypeptides of the fusion protein are arranged, and therefore, the order of the encoding nucleic acid fragments in the nucleic acid vector, can be altered without affecting immunogenicity of the fusion polypeptides proteins and the utility of the composition. For example, the ETA(dII)-encoding DNA sequences may be located 5' or 3' to the target antigen-encoding sequences. In one embodiment, these polypeptide-encoding nucleic acid domains are in-frame so that the DNA construct encodes a recombinant fusion polypeptide in which the antigen is located N- terminal to the ETA(dII)-derived polypeptide.

The immunogenic compositions of the present invention include, (a) an antigenic peptide comprising one or more epitopes of a single antigen or multiple different antigens, and (b) a translocation polypeptide such as ETA(dII). In addition to the specific antigens and vectors employed in the Examples, the present invention is intended to encompass a vector such as naked RNA, self replicating RNA replicons and viruses including vaccinia, adenoviruses, adeno-associated virus (AAV), lentiviruses and RNA alphaviruses.

In addition to the translocation polypeptide, the immunogenic nucleic acid construct of the present invention may, optionally, also include

(a) an additional antigen targeting or processing signal such as a protein that promotes intercellular transport, *e.g.*, VP22 protein from herpes simplex virus and related herpes viruses (see, for example, commonly assigned International patent application published as WO 02/09645, 07-Feb-2002, incorporated by reference in its entirety); an endoplasmic reticulum chaperone polypeptide such as calreticulin, ER60, GRP94 or gp96, well-characterized ER chaperone polypeptide that representatives of the heat shock protein (*e.g.*, HSP90) family of stress-induced proteins. (See, co-pending commonly assigned

International patent application published as WO 02/09645, 14-FEB-2002, incorporated by reference in its entirety; see also Argon (1999) *Semin. Cell Dev..Biol.* 10:495-505; Sastry (1999) *J.* Biol. Chem. *274*:12023-12035; Nicchitta (1998) *Curr. Opin. Immunol.* 10:103-109; U.S. Patent 5,981,706).

(b) an immunostimulatory cytokine, preferably one that targets APCs, preferably DCs, such as granulocyte macrophage colony stimulating factor (GM-CSF), or active fragments or domains thereof; and

(c) a costimulatory signal, such as a B7 family protein, including B7-DC (see commonly assigned U.S. patent application Serial No. 09/794,210), B7.1, B7.2, soluble CD40, *etc.*).

(For description of some of the foregoing, see, for example, commonly owned International patent applications PCT/US01/23966, PCT/US01/24134, PCTUS/00/41422))

Naked DNA vaccines represent an attractive approach for generating antigen-specific immunity because of their stability and simplicity of delivery. Concerns with DNA vaccines include potential integration into the host genome, cell transformation, and limited potency. The use of DNA-based alphaviral RNA replicons ("suicidal DNA vectors"), as disclosed herein, may alleviate concerns surrounding DNA integration or cell transformation since suicidal DNA vectors eventually cause lysis of the cells they transfect.

To further improve the potency of suicidal DNA vaccines, ETA(dII) is linked to an antigen such as E7 as a model antigen, using DNA-based Semliki Forest virus (SFV) RNA vector, pSCA1. This suicidal DNA vaccine containing ETA(dII)/E7/fusion DNA produces significantly greater E7-specific T cell-mediated immune response in mice than do vaccines containing the wild type E7 DNA alone. Importantly, this fusion converts a less effective vaccine into one with significant therapeutic potency against established E7-expressing metastatic tumors. The antitumor effect is dependent upon CD8+ T cells. Thus, linkage of ETA(dII) to an antigen enhances the potency of a suicidal DNA vaccine.

In the methods of the invention, the chimeric polypeptide or nucleic acid that encodes it are employed to induce or enhance immune responses. In one embodiment, the compositions of the invention synergistically enhance immune responses and antitumor effects through both immunological and anti-angiogenic mechanisms.

The experiments described herein demonstrate that the methods of the invention can enhance a cellular immune response, particularly, tumor-destructive CTL reactivity, induced by a

DNA vaccine encoding an epitope of a human pathogen. Human HPV-16 E7 was used as a model antigen for vaccine development because human papillomaviruses (HPVs), particularly HPV-16, are associated with most human cervical cancers. The oncogenic HPV protein E7 is important in the induction and maintenance of cellular transformation and co-expressed in most HPV-containing cervical cancers and their precursor lesions. Therefore, cancer vaccines, such as the compositions of the invention, that target E7 can be used to control of HPV-associated neoplasms (Wu (1994) *Curr. Opin. Immunol.* 6:746-754).

In one embodiment, the antigen (*e.g.*, the MHC class I-binding peptide epitope) is derived from a pathogen, *e.g.*, it comprises a peptide expressed by a pathogen. The pathogen can be a virus, such as, *e.g.*, a papilloma virus, a herpesvirus, a retrovirus (*e.g.*, an immunodeficiency virus, such as HIV-1), an adenovirus, and the like. The papilloma virus can be a human papilloma virus; for example, the antigen (*e.g.*, the Class I-binding peptide) can be derived from an HPV-16 E7 polypeptide. In one embodiment, the HPV-16 E7 polypeptide is substantially non-oncogenic, *i.e.*, it does not bind retinoblastoma polypeptide (pRB) or binds pRB with such low affinity that the HPV-16 E7 polypeptide is effectively non-oncogenic when expressed or delivered *in vivo*.

In alternative embodiments, the pathogen is a bacteria, such as *Bordetella pertussis*; *Ehrlichia chaffeensis*; *Staphylococcus aureus*; *Toxoplasma gondii*; *Legionella pneumophila*; *Brucella suis*; *Salmonella enterica*; *Mycobacterium avium*; *Mycobacterium tuberculosis*; *Listeria monocytogenes*; *Chlamydia trachomatis*; *Chlamydia pneumoniae*; *Rickettsia rickettsii*; or, a fungus, such as, *e.g.*, *Paracoccidioides brasiliensis*; or other pathogen, *e.g.*, *Plasmodium falciparum*.

In another embodiment, the MHC class I-binding peptide epitope is derived from a tumor cell. The tumor cell-derived peptide epitope can comprise a tumor associated antigen, *e.g.*, a tumor specific antigen, such as, *e.g.*, a HER-2/neu antigen.

In one embodiment, the isolated or recombinant nucleic acid molecule is operatively linked to a promoter, such as, *e.g.*, a constitutive, an inducible or a tissue-specific promoter. The promoter can be expressed in any cell, including cells of the immune system, including, *e.g.*, antigen presenting cells (APCs), *e.g.*, in a constitutive, an inducible or a tissue-specific manner.

In alternative embodiments, the APCs are dendritic cells, keratinocytes, astrocytes, monocytes, macrophages, B lymphocytes, a microglial cell, or activated endothelial cells, and the like.

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art of this invention. As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

The term "antigen" or "immunogen" as used herein refers to a compound or composition comprising a peptide, polypeptide or protein which is "antigenic" or "immunogenic" when administered (or expressed *in vivo* by an administered nucleic acid, *e.g.*, a DNA vaccine) in an appropriate amount (an "immunogenically effective amount"), which means that it is capable of inducing, eliciting, augmenting or boosting a cellular and/or humoral immune response either alone or in combination or linked or fused to another substance (which can be administered at once or over several intervals). An immunogenic composition can comprise an antigenic peptide of at least about 5 amino acids, a peptide of 10 amino acids in length, a polypeptide fragment of 15 amino acids in length, 20 amino acids in length or longer. Smaller immunogens may require presence of a "carrier" polypeptide *e.g.*, as a fusion protein, aggregate, conjugate or mixture, preferably linked (chemically or otherwise) to the immunogen. The immunogen can be recombinantly expressed from a vaccine vector, which can be naked DNA comprising the immunogen's coding sequence operably linked to a promoter, *e.g.*, an expression cassette as described herein. The immunogen includes one or more antigenic determinants or epitopes which may vary in size from about 3 to about 15 amino acids. The "antigen"-encoding portion or domain of the nucleic acid may comprise one or more natural epitopes or variants thereof, which may be derived from a single protein (*e.g.*, HPV-E7) or multiple proteins (*e.g.*, HPV-E6, any other tumor-associated antigen such as those associated with leukemias, lymphomas, melanomas, adenocarcinomas, *etc.*

The term "epitope" as used herein refers to an antigenic determinant or antigenic site that interacts with an antibody or a T cell receptor (TCR), *e.g.*, the MHC class I-binding peptide compositions (or expressed products of the nucleic acid compositions of the invention) used in the methods of the invention. An "antigen" is a molecule or chemical structure that either induces an immune response or is specifically recognized or bound by the product or mediator of an immune response, such as an antibody or a CTL. The specific conformational or

stereochemical "domain" to which an antibody or a TCR bind is an "antigenic determinant" or "epitope." TCRs bind to peptide epitopes which are physically associated with a third molecule, a major histocompatibility complex (MHC) class I or class II protein.

The term "recombinant" refers to (1) a nucleic acid or polynucleotide synthesized or otherwise manipulated *in vitro*, (2) methods of using recombinant DNA technology to produce gene products in cells or other biological systems, or (3) a polypeptide encoded by a recombinant nucleic acid. For example, the ETA(dII)-encoding nucleic acid or polypeptide, the nucleic acid encoding an MHC class I-binding peptide epitope (antigen) or the peptide itself can be recombinant. "Recombinant means" includes ligation of nucleic acids having various coding regions or domains or promoter sequences from different sources into a single unit in the form of an expression cassette or vector for expression of the coding sequences in the vectors resulting in production of the encoded polypeptide.

The term "self-replicating RNA replicon" refers to a construct based on an RNA viruses, such as alphavirus genome RNAs (*e.g.*, Sindbis virus, Semliki Forest virus, *etc.*), that have been engineered to allow expression of heterologous RNAs and proteins. These recombinant vectors are self-replicating ("replicons") which can be introduced into cells as naked RNA or DNA, as described in detail in co-pending, commonly assigned U.S. and PCT patent applications by the present inventors, having serial numbers 10/060,274, and PCT/US02/_____, both filed on 10 February 2002, and entitled *"Superior Molecular Vaccine Based on Self-Replicating RNA, Suicidal DNA or Naked DNA Vector, that Links Antigen with Polypeptide that Promotes Antigen Presentation."* In one embodiment, the self-replicating RNA replicon comprises a Sindbis virus self-replicating RNA vector SINrep5, which is described in detail in U.S. Patent No. 5,217,879.

The present compositions and methods have utility not only in the context of treating or preventing the development or progression of a disease state, for example cancer or infectious disease (as described below), but also for enhancing specific immune responses to an antigen of choice. Measurement of such enhanced immune responses can serve as the read-out of a screening test for new immunogenic or vaccine compositions being developed. These compositions and methods also serve to improve the efficiency of inducing antigen specific CTLs and for their isolation and propagation. Such CTLs may be used for production of their secreted products, cell surface products (*e.g.*, T cell receptors) or intracellular products, for

cellular therapy *in vivo*, or for further study and understanding of these cells, their products, their activation properties, *etc.*

SEQUENCES OF POLYPEPTIDES AND NUCLEIC ACIDS

The section that follows lists the sequences of the ETA(dII) polypeptides alone or in fusion with E7 antigen, the nucleic acids encoding some of these peptides and nucleic acids of the vectors into which the sequences encoding these polypeptides are cloned. The complete coding sequence for *Pseudomonas aeruginosa* exotoxin type A (ETA) - SEQ ID NO:1 - GenBank Accession No. K01397, is shown below:

```
1     ctgcagctgg tcaggccgtt tccgcaacgc ttgaagtcct ggccgatata ccggcagggc
61    cagccatcgt tcgacgaata aagccacctc agccatgatg ccctttccat ccccagcgga
121   accccgacat ggacgccaaa gccctgctcc tcggcagcct ctgcctggcc gccccattcg
181   ccgacgcggc gacgctcgac aatgctctct ccgcctgcct cgccgcccgg ctcggtgcac
241   cgcacacggc ggagggccag ttgcacctgc cactcaccct tgaggcccgg cgctccaccg
301   gcgaatgcgg ctgtacctcg gcgctggtgc gatatcggct gctggccagg ggcgccagcg
361   ccgacagcct cgtgcttcaa gagggctgct cgatagtcgc caggacacgc cgcgcacgct
421   gaccctggcg gcggacggcg gcttggcgag cggccgcgaa ctggtcgtca ccctgggttg
481   tcaggcgcct gactgacagg ccgggctgcc accaccaggc cgagatggac gccctgcatg
541   tatcctccga tcggcaagcc tcccgttcgc acattcacca ctctgcaatc cagttcataa
601   atcccataaa agcccttctc cgctccccgc cagcctcccc gcatcccgca ccctagacgc
661   ccgccgctc tccgccggct cgcccgacaa gaaaaaccaa ccgctcgatc agcctcatcc
721   ttcacccatc acaggagcca tcgcgatgca cctgataccc cattggatcc ccctggtcgc
781   cagcctcggc ctgctcgccg gcggctcgtc cgcgtcgcc gccgaggaag ccttcgacct
841   ctggaacgaa tgcgccaaag cctgcgtgct cgacctcaag gacggcgtgc gttccagccg
901   catgagcgtc gacccggcca tcgccgacac caacggccag ggcgtgctgc actactccat
961   ggtcctggag ggcggcaacg acgcgctcaa gctggccatc gacaacgccc tcagcatcac
1021  cagcgacggc ctgaccatcc gcctcgaagg cggcgtcgag ccgaacaagc cggtgcgcta
1081  cagctacacg cgccaggcgc gcggcagttg gtcgctgaac tggctggtac cgatcggcca
1141  cgagaagccc tcgaacatca aggtgttcat ccacgaactg aacgccggca accagctcag
1201  ccacatgtcg ccgatctaca ccatcgagat gggcgacgag ttgctggcga agctggcgcg
1261  cgatgccacc ttcttcgtca gggcgcacga gagcaacgag atgcagccga cgctcgccat
1321  cagccatgcc ggggtcagcg tggtcatggc ccagacccag ccgcgccggg aaaagcgctg
1381  gagcgaatgg gccagcggca aggtgttgtg cctgctcgac ccgctggacg gggtctacaa
1441  ctacctcgcc cagcaacgct gcaacctcga cgatacctgg gaaggcaaga tctaccgggt
1501  gctcgccggc aacccggcga agcatgacct ggacatcaaa cccacggtca tcagtcatcg
1561  cctgcacttt cccgagggcg gcagcctggc cgcgctgacc gcgcaccagg cttgccacct
1621  gccgctggag acttcaccc gtcatcgcca gccgcggcgc tgggaacaac tggaggcagtg
1681  cggctatccg gtgcagcggc tggtcgccct ctacctggcg gcggcggctgt cgtggaacca
1741  ggtcgaccag gtgatccgca acgcctggc cagccccggc agcggcggcg acctgggcga
1801  agcgatccgc gagcagccgg agcaggcccg tctggcctg accctgacgg ccgccagag
1861  cgagcgcttc gtccggcagg gcaccggcaa cgacgaggcc ggcgcggcca acgccgacgt
1921  ggtgagcctg acctgccgg tcgccgccgg tgaatgcgcg ggcccggcgg acagcggcga
1981  cgcctgctg gagcgcaact atcccactgg cgcggagttc ctcggcgacg gcggcgacgt
2041  cagcttcagc acccgcggca cgcagaactg gacggtggag cggctgctcc aggcgcaccg
2101  ccaactggag gagcgcggct atgtgttcgt cggctaccac ggcaccttcc tcgaagcggc
2161  gcaaagcatc gtcttcggcg gggtgcgcgc gcgcagccag gacctcgacg cgatctggcg
2221  cggtttctat atcgccggcg atccggcgct ggcctacggc tacgcccagg accaggaacc
2281  cgacgcacgc ggccggatcc gcaacggtgc cctgctgcgg gtctatgtgc cgcgctcgag
2341  cctgccgggc ttctaccgca ccagcctgac cctggccgcg ccggaggcgg cgggcgaggt
2401  cgaacggctg atcggccatc cgctgccgct gcgcctggac gccatcaccg gccccgagga
2461  ggaaggcggg cgcctggaga ccattctcgg ctggccgctg gccgagcgca ccgtggtgat
2521  tccctcggcg atccccaccg acccgcgcaa cgtcggcggc gacctcgacc cgtccagcat
2581  ccccgacaag gaacaggcga tcagcgccct gccggactac gccagccagc ccggcaaacc
2641  gccgcgcgag gacctgaagt aactgccgcg accggccggc tccctcgca gcagccggcc
2701  ttctcggggc ctggccatac atcaggtttt cctgatgcca gcccaatcga atatgaattc  2760
```

The amino acid sequence of ETA (SEQ ID NO:2), GenBank Accession No. K01397, is shown below

```
MHLIPHWIPL VASLGLLAGG SSASAAEEAF DLWNECAKAC VLDLKDGVRS SRMSVDPAIA   60
DTNGQGVLHY SMVLEGGNDA LKLAIDNALS ITSDGLTIRL EGGVEPNKPV RYSYTRQARG  120
SWSLNWLVPI GHEKPSNIKV FIHELNAGNQ LSHMSPIYTI EMGDELLAKL ARDATFFVRA  180
HESNEMQPTL AISHAGVSVV MAQTQPRREK RWSEWASGKV LCLLDPLDGV YNYLAQQRCN  240
LDDTWEGKIY RVLAGNPAKH DLDIKPTVIS HRLHFPEGGS LAALTAHQAC HLPLETFTRH  300
RQPRGWEQLE QCGYPVQRLV ALYLAARLSW NQVDQVIRNA LASPGSGGDL GEAIREQPEQ  360
ARLALTLAAA ESERFVRQGT GNDEAGAANA DVVSLTCPVA AGECAGPADS GDALLERNYP  420
TGAEFLGDGG DVSFSTRGTQ NWTVERLLQA HRQLEERGYV FVGYHGTFLE AAQSIVFGGV  480
RARSQDLDAI WRGFYIAGDP ALAYGYAQDQ EPDARGRIRN GALLRVYVPR SSLPGFYRTS  540
LTLAAPEAAG EVERLIGHPL PLRLDAITGP EEEGGRLETI LGWPLAERTV VIPSAIPTDP  600
RNVGGDLDPS SIPDKEQAIS ALPDYASQPG KPPREDLK                          638
```

Residues 1-25 (italicized) represent the signal peptide; the start of the mature polypeptide is shown as a bold/underlined A. The mature polypeptide is residues 26-638 of SEQ ID NO:2. The ETA(dII) translocation domain (underscored above) spans residues 247-417 of the mature polypeptide (corresponding to residues 272-442 of SEQ ID NO:2) and is presented below separately as SEQ ID NO:3.

```
RLHFPEGGSL AALTAHQACH LPLETFTRHR QPRGWEQLEQ CGYPVQRLVA LYLAARLSWN   60
QVDQVIRNAL ASPGSGGDLG EAIREQPEQA RLALTLAAAE SERFVRQGTG NDEAGAANAD  120
VVSLTCPVAA GECAGPADSG DALLERNYPT GAEFLGDGGD VSFSTRGTQN W           171
```

The sequences shown below (nucleotide is SEQ ID NO:4 and amino acid is SEQ ID NO:5) are the construct in which ETA(dII) is fused to the HPV-16 E7 polypeptide. The ETA(dII) sequence appears in plain font, extra codons from pcDNA3 are italicized; those between the ETA(dII) and E7 sequence are also bolded (and result in the interposition of two amino acids between ETA(dII) and E7. The E7 sequence is underscored. The E7 sequence ends in Gln.

```
1/1                                     31/11

atg cgc ctg cac ttt ccc gag ggc ggc agc ctg gcc gcg ctg acc gcg cac cag gct tgc
Met arg leu his phe pro glu gly gly ser leu ala ala leu thr ala his gln ala cys
61/21                                   91/31
cac ctg ccg ctg gag act ttc acc cgt cat cgc cag ccg cgc ggc tgg gaa caa ctg gag
his leu pro leu glu thr phe thr arg his arg gln pro arg gly trp glu gln leu glu
121/41                                  151/51
cag tgc ggc tat ccg gtg cag cgg ctg gtc gcc ctc tac ctg gcg gcg cgg ctg tcg tgg
gln cys gly tyr pro val gln arg leu val ala leu tyr leu ala ala arg leu ser trp
181/61                                  211/71
aac cag gtc gac cag gtg atc cgc aac gcc ctg gcc agc ccc ggc agc ggc ggc gac ctg
asn gln val asp gln val ile arg asn ala leu ala ser pro gly ser gly gly asp leu
241/81                                  271/91
ggc gaa gcg atc cgc gag cag ccg gag cag gcc cgt ctg gcc ctg acc ctg gcc gcc gcc
gly glu ala ile arg glu gln pro glu gln ala arg leu ala leu thr leu ala ala ala
```

```
301/101                                 331/111
gag agc gag cgc ttc gtc cgg cag ggc acc ggc aac gac gag gcc ggc gcg gcc aac gcc
glu ser glu arg phe val arg gln gly thr gly asn asp glu ala gly ala ala asn ala
361/121                                 391/131
gac gtg gtg agc ctg acc tgc ccg gtc gcc gcc ggt gaa tgc gcg ggc ccg gcg gac agc
asp val val ser leu thr cys pro val ala ala gly glu cys ala gly pro ala asp ser
421/141                                 451/151
ggc gac gcc ctg ctg gag cgc aac tat ccc act ggc gcg gag ttc ctc ggc gac ggc ggc
gly asp ala leu leu glu arg asn tyr pro thr gly ala glu phe leu gly asp gly gly
481/161                                 511/171
gac gtc agc ttc agc acc cgc ggc acg cag aac gaa ttc atg cat gga gat aca cct aca
asp val ser phe ser thr arg gly thr gln asn glu phe met his gly asp thr pro thr
541/181                                 571/191
ttg cat gaa tat atg tta gat ttg caa cca gag aca act gat ctc tac tgt tat gag caa
leu his glu tyr met leu asp leu gln pro glu thr thr asp leu tyr cys tyr glu gln
601/201                                 631/211
tta aat gac agc tca gag gag gag gat gaa ata gat ggt cca gct gga caa gca gaa ccg
leu asn asp ser ser glu glu glu asp glu ile asp gly pro ala gly gln ala glu pro
661/221                                 691/231
gac aga gcc cat tac aat att gta acc ttt tgt tgc aag tgt gac tct acg ctt cgg ttg
asp arg ala his tyr asn ile val thr phe cys cys lys cys asp ser thr leu arg leu
721/241                                 751/251
tgc gta caa agc aca cac gta gac att cgt act ttg gaa gac ctg tta atg ggc aca cta
cys val gln ser thr his val asp ile arg thr leu glu asp leu leu met gly thr leu
781/261                                 811/271
gga att gtg tgc ccc atc tgt tct caa gga tcc gag ctc ggt acc aag ctt aag ttt aaa
gly ile val cys pro ile cys ser gln gly ser glu leu gly thr lys leu lys phe lys
841/281
ccg ctg atc agc ctc gac tgt gcc ttc tag
pro leu ile ser leu asp cys ala phe AMB
```

Compared to the GenBank sequence of E7 (SEQ ID NO:6 & 7) shown below, three C-terminal amino acids have been deleted. The HPV E7 sequence (nucleotide sequence is SEQ ID NO:6 and amino acid sequence is SEQ ID NO:7) is shown below:

```
1/1                                     31/11
atg cat gga gat aca cct aca ttg cat gaa tat atg tta gat ttg caa cca gag aca act
Met his gly asp thr pro thr leu his glu tyr met leu asp leu gln pro glu thr thr
61/21                                   91/31
gat ctc tac tgt tat gag caa tta aat gac agc tca gag gag gag gat gaa ata gat ggt
asp leu tyr cys tyr glu gln leu asn asp ser ser glu glu glu asp glu ile asp gly
121/41                                  151/51
cca gct gga caa gca gaa ccg gac aga gcc cat tac aat att gta acc ttt tgt tgc aag
pro ala gly gln ala glu pro asp arg ala his tyr asn ile val thr phe cys cys lys
181/61                                  211/71
tgt gac tct acg ctt cgg ttg tgc gta caa agc aca cac gta gac att cgt act ttg gaa
cys asp ser thr leu arg leu cys val gln ser thr his val asp ile arg thr leu glu
241/81                                  271/91
gac ctg tta atg ggc aca cta gga att gtg tgc ccc atc tgt tct cag gat aag ctt
asp leu leu met gly thr leu gly ile val cys pro ile cys ser gln asp lys leu
```

The sequence of the **pcDNA3 plasmid vector** (SEQ ID NO:8) is:

```
GACGGATCGG GAGATCTCCC GATCCCCTAT GGTCGACTCT CAGTACAATC TGCTCTGATG CCGCATAGTT AAGCCAGTAT
CTGCTCCCTG CTTGTGTGTT GGAGGTCGCT GAGTAGTGCG CGAGCAAAAT TTAAGCTACA ACAAGGCAAG GCTTGACCGA
CAATTGCATG AAGAATCTGC TTAGGGTTAG GCGTTTTGCG CTGCTTCGCG ATGTACGGGC CAGATATACG CGTTGACATT
GATTATTGAC TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTTCAT AGCCCATATA TGGAGTTCCG CGTTACATAA
CTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC CCCGCCCATT GACGTCAATA ATGACGTATG TTCCCATAGT
AACGCCAATA GGGACTTTCC ATTGACGTCA ATGGGTGGAC TATTTACGGT AAACTGCCCA CTTGGCAGTA CATCAAGTGT
ATCATATGCC AAGTACGCCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT ATGCCCAGTA CATGACCTTA
TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA TCGCTATTAC CATGGTGATG CGGTTTTGGC AGTACATCAA
TGGGCGTGGA TAGCGGTTTG ACTCACGGGG ATTTCCAAGT CTCCACCCCA TTGACGTCAA TGGGAGTTTG TTTTGGCACC
AAAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGGCG GTAGGCGTGT ACGGTGGGAG
GTCTATATAA GCAGAGCTCT CTGGCTAACT AGAGAACCCA CTGCTTACTG GCTTATCGAA ATTAATACGA CTCACTATAG
GGAGACCCAA GCTGGCTAGC GTTTAAACGG GCCCTCTAGA CTCGAGCGGC CGCCACTGTG CTGGATATCT GCAGAATTCC
ACCACACTGG ACTAGTGGAT CCGAGCTCGG TACCAAGCTT AAGTTTAAAC CGCTGATCAG CCTCGACTGT GCCTTCTAGT
TGCCAGCCAT CTGTTGTTTG CCCCTCCCCC GTGCCTTCCT TGACCCTGGA AGGTGCCACT CCCACTGTCC TTTCCTAATA
AAATGAGGAA ATTGCATCGC ATTGTCTGAG TAGGTGTCAT TCTATTCTGG GGGGTGGGGT GGGGCAGGAC AGCAAGGGGG
AGGATTGGGA AGACAATAGC AGGCATGCTG GGGATGCGGT GGGCTCTATG GCTTCTGAGG CGGAAAGAAC CAGCTGGGGC
TCTAGGGGGT ATCCCCACGC GCCCTGTAGC GGCGCATTAA GCGCGGCGGG TGTGGTGGTT ACGCGCAGCG TGACCGCTAC
ACTTGCCAGC GCCCTAGCGC CCGCTCCTTT CGCTTTCTTC TCGCCACGTT CGCCGGCTTT CCCCGTCAAG CTCTAAATCG
CTCTAAATCG GGGCATCCCT TTAGGGTTCC GATTTAGTGC TTTACGGCAC CTCGACCCCA AAAAACTTGA TTAGGGTGAT
GGTTCACGTA GTGGGCCATC GCCCTGATAG ACGGTTTTTC GCCCTTTGAC GCTGGAGTCC ACGTTCTTTA ATAGTGGACT
CTTGTTCCAA ACTGGAACAA CACTCAACCC TATCTCGGTC TATTCTTTTG ATTTATAAGG GATTTTGGGG ATTTCGGCCT
ATTGGTTAAA AAATGAGCTG ATTTAACAAA AATTTAACGC GAATTAATTC TGTGGAATGT GTGTCAGTTA GGGTGTGGAA
AGTCCCCAGG CTCCCCAGGC AGGCAGAAGT ATGCAAAGCA TGCATCTCAA TTAGTCAGCA ACCAGGTGTG GAAAGTCCCC
AGGCTCCCCA GCAGGCAGAA GTATGCAAAG CATGCATCTC AATTAGTCAG CAACCATAGT CCCGCCCCTA ACTCCGCCCA
TCCCGCCCCT AACTCCGCCC AGTTCCGCCC CATTCTCCGC CCATGGCTGA CTAATTTTTT TTATTTATGC AGAGGCCGAG
GCCGCCTCTG CCTCTGAGCT ATTCCAGAAG TAGTGAGGAG GCTTTTTTGG AGGCCTAGGC TTTTGCAAAA AGCTCCCGGG
AGCTTGTATA TCCATTTTCG GATCTGATCA AGAGACAGGA TGAGGATCGT TTCGCATGAT TGAACAAGAT GGATTGCACG
CAGGTTCTCC GGCCGCTTGG GTGGAGAGGC TATTCGGCTA TGACTGGGCA CAACAGACAA TCGGCTGCTC TGATGCCGCC
GTGTTCCGGC TGTCAGCGCA GGGGCGCCCG GTTCTTTTTG TCAAGACCGA CCTGTCCGGT GCCCTGAATG AACTGCAGGA
CGAGGCAGCG CGGCTATCGT GGCTGGCCAC GACGGGCGTT CCTTGCGCAG CTGTGCTCGA CGTTGTCACT GAAGCGGGAA
GGGACTGGCT GCTATTGGGC GAAGTGCCGG GGCAGGATCT CCTGTCATCT CACCTTGCTC CTGCCGAGAA AGTATCCATC
ATGGCTGATG CAATCGCGCG CGCGATCAGG TTGGTCCGTC CTACGTGCCC ATTCGACCAC CAAGCGAAAC ATCGCATCGA
GCGAGCACGT ACTCGGATGG AAGCCGGTCT TGTCGATCAG GATGATCTGG ACGAAGAGCA TCAGGGGCTC GCGCCAGCCG
AACTGTTCGC CAGGCTCAAG GCGCGCATGC CCGACGGCGA GGATCTCGTC GTGACCCATG GCGATGCCTG CTTGCCGAAT
ATCATGGTGG AAAATGGCCG CTTTTCTGGA TTCATCGACT GTGGCCGGCT GGGTGTGGCG GACCGCTATC AGGACATAGC
GTTGGCTACC CGTGATATTG CTGAAGAGCT TGGCGGCGAA TGGGCTGACC GCTTCCTCGT GCTTTACGGT ATCGCCGCTC
CCGATTCGCA GCGCATCGCC TTCTATCGCC TTCTTGACGA GTTCTTCTGA GCGGGACTCT GGGGTTCGAA ATGACCGACC
AAGCGACGCC CAACCTGCCA TCACGAGATT TCGATTCCAC CGCCGCCTTC TATGAAAGGT TGGGCTTCGG AATCGTTTTC
CGGGACGCCG GCTGGATGAT CCTCCAGCGC GGGGATCTCA TGCTGGAGTT CTTCGCCCAC CCCAACTTGT TTATTGCAGC
TTATAATGGT TACAAATAAA GCAATAGCAT CACAAATAAG CATTTTTTTC ACTGCATTCT AGTTGTGGTT TGTCCAAACT
CATCAATGTA TCTTATCATG TCTGTATACC GTCGACCTCT AGCTAGAGCT TGGCGTAATC ATGGTCATAG CTGTTTCCTG
TGTGAAATTG TTATCCGCTC ACAATTCCAC ACAACATACG AGCCGGAAGC ATAAAGTGTA AAGCCTGGGG TGCCTAATGA
GTGAGCTAAC TCACATTAAT TGCGTTGCGC TCACTGCCCG CTTTCCAGTC GGGAAACCTG TCGTGCCAGC TGCATTAATG
AATCGGCCAA CGCGCGGGGA GAGGCGGTTT GCGTATTGGG CGCTCTTCCG CTTCCTCGCT CACTGACTCG CTGCGCTCGG
TCGTTCGGCT GCGGCGAGCG GTATCAGCTC ACTCAAAGGC GGTAATACGG TTATCCACAG AATCAGGGGA TAACGCAGGA
AAGAACATGT GAGCAAAAGG CCAGCAAAAG GCCAGGAACC GTAAAAAGGC CGCGTTGCTG GCGTTTTTCC ATAGGCTCCG
CCCCCCTGAC GAGCATCACA AAAATCGACG CTCAAGTCAG AGGTGGCGAA ACCCGACAGG ACTATAAAGA TACCAGGCGT
TTCCCCCTGG AAGCTCCCTC GTGCGCTCTC CTGTTCCGAC CCTGCCGCTT ACCGGATACC TGTCCGCCTT TCTCCCTTCG
GGAAGCGTGG CGCTTTCTCA ATGCTCACGC TGTAGGTATC TCAGTTCGGT GTAGGTCGTT CGCTCCAAGC TGGGCTGTGT
GCACGAACCC CCCGTTCAGC CCGACCGCTG CGCCTTATCC GGTAACTATC GTCTTGAGTC CAACCCGGTA AGACACGACT
TATCGCCACT GGCAGCAGCC ACTGGTAACA GGATTAGCAG AGCGAGGTAT GTAGGCGGTG CTACAGAGTT CTTGAAGTGG
CTTGAAGTGG TGGCCTAACT ACGGCTACAC TAGAAGGACA GTATTTGGTA TCTGCGCTCT GCTGAAGCCA GTTACCTTCG
GAAAAAGAGT TGGTAGCTCT TGATCCGGCA AACAAACCAC CGCTGGTAGC GGTGGTTTTT TTGTTTGCAA GCAGCAGATT
ACGCGCAGAA AAAAGGATCT CAAGAAGATC CTTTGATCTT TTCTACGGG GTCTGACGCT CAGTGGAACG AAAACTCACG
TTAAGGGATT TTGGTCATGA GATTATCAAA AAGGATCTTC ACCTAGATCC TTTTAAATTA AAAATGAAGT TTTAAATCAA
TCTAAAGTAT ATATGAGTAA ACTTGGTCTG ACAGTTACCA ATGCTTAATC AGTGAGGCAC CTATCTCAGC GATCTGTCTA
TTTCGTTCAT CCATAGTTGC CTGACTCCCC GTCGTGTAGA TAACTACGAT ACGGGAGGGC TTACCATCTG GCCCCAGTGC
TGCAATGATA CCGCGAGACC CACGCTCACC GGCTCCAGAT TTATCAGCAA TAAACCAGCC AGCCGGAAGG GCCGAGCGCA
GAAGTGGTCC TGCAACTTTA TCCGCCTCCA TCCAGTCTAT TAATTGTTGC CGGGAAGCTA GAGTAAGTAG TTCGCCAGTT
AATAGTTTGC GCAACGTTGT TGCCATTGCT ACAGGCATCG TGGTGTCACG CTCGTCGTTT GGTATGGCTT CATTCAGCTC
CGGTTCCCAA CGATCAAGGC GAGTTACATG ATCCCCCATG TTGTGCAAAA AAGCGGTTAG CTCCTTCGGT CCTCCGATCG
TTGTCAGAAG TAAGTTGGCC GCAGTGTTAT CACTCATGGT TATGGCAGCA CTGCATAATT CTCTTACTGT CATGCCATCC
GTAAGATGCT TTTCTGTGAC TGGTGAGTAC TCAACCAAGT CATTCTGAGA ATAGTGTATG CGGCGACCGA GTTGCTCTTG
CCCGGCGTCA ATACGGGATA ATACCGCGCC ACATAGCAGA ACTTTAAAAG TGCTCATCAT TGGAAAACGT TCTTCGGGGC
GAAAACTCTC AAGGATCTTA CCGCTGTTGA GATCCAGTTC GATGTAACCC ACTCGTGCAC CCAACTGATC TTCAGCATCT
TTTACTTTCA CCAGCGTTTC TGGGTGAGCA AAAACAGGAA GGCAAAATGC CGCAAAAAAG GGAATAAGGG CGACACGGAA
ATGTTGAATA CTCATACTCT TCCTTTTTCA ATATTATTGA AGCATTTATC AGGGTTATTG TCTCATGAGC GGATACATAT
TTGAATGTAT TTAGAAAAAT AAACAAATAG GGGTTCCGCG CACATTTCCC CGAAAAGTGC CACCTGACGT C
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The nucleic acid sequence of plasmid construct pcDNA3-ETA(dII)/E7 (SEQ ID NO:9) is shown below. ETA(dII)/E7 is ligated in the EcoRI/BamHI sites of pcDNA3 vector. The nucleotides encoding ETA(dII)/E7 are shown in lower case bold.

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          10        20        30        40        50        60        70        80
   1 GACGGATCGG GAGATCTCCC GATCCCCTAT GGTCGACTCT CAGTACAATC TGCTCTGATG CCGCATAGTT AAGCCAGTAT  80
  81 CTGCTCCCTG CTTGTGTGTT GGAGGTCGCT GAGTAGTGCG CGAGCAAAAT TTAAGCTACA ACAAGGCAAG GCCTATGGCA 160
 161 CAATTGCATG AAGAATCTGC TTAGGGTTAG GCGTTTTGCG CTGCTTCGCG ATGTACGGGC CAGATATACG CGTTGACATT 240
 241 GATTATTGAC TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTTCAT AGCCCATATA TGGAGTTCCG CGTTACATAA 240
 321 CTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC CCCGCCCATT GACGTCAATA ATGACGTATG TTCCCATAGT 400
 401 AACGCCAATA GGGACTTTCC ATTGACGTCA ATGGGTGGAC TATTTACGGT AAACTGCCCA CTTGGCAGTA CATCAAGTGT 480
 481 ATCATATGCC AAGTACGCCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT ATGCCCAGTA CATGACCTTA 560
 561 TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA TCGCTATTAC CATGGTGATG CGGTTTTGGC AGTACATCAA 640
 641 TGGGCGTGGA TAGCGGTTTG ACTCACGGGG ATTTCCAAGT CTCCACCCCA TTGACGTCAA TGGGAGTTTG TTTTGGCACC 720
 721 AAAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGGCG GTAGGCGTGT ACGGTGGGAG 800
 801 GTCTATATAA GCAGAGCTCT CTGGCTAACT AGAGAACCCA CTGCTTACTG GCTTATCGAA ATTAATACGA CTCACTATAG 880
 881 GGAGACCCAA GCTGGCTAGC GTTTAAACGG GCCCTCTAGA CTCGAGCGGC CGCCACTGTG CTGGATATCT GCAGAATTCa 960
 961 tggcctgca ctttccgagg gggggcagcc tggccgcgct gaccgcgcac caggcttgcc acctgccgct ggagacttcc 1040
1041 accgtatcc gcgccttttc attgacgtca aaatcgagcc agtgcgctca tccggtgcag cggctggtcg ccctctacct 1120
1121 gggggcggcg ctgtctgtga accaggtcga ccagatgatc cgcaagcgcc tggccaagcc cggcaagccc cggcgagcgg gccgacctgg 1200
1201 gcgaaggcat ccgcgagcag ccggtcgagc ccgtcctgcc ccgaccctg gcgccgccg agagcgagcg ctcgtccgg 1280
1281 cagggcaccg gcaacgacga ggccgcgcg gccaacgccg acgtcgatgg cctgacctgc ccggtcgccg ccggtgaatg 1360
1361 cgcgggccg gcgacgcgg gcgacgccct gctggacggc aactatccca ctggcgcga gtccctcgc gacggcggcg 1440
1441 acgtcagctt cagcaccgc ggcacgcaga acgaattcat gcatggagat acacctacat tgcatgaata tatgttagat 1520
1521 ttgcaaccag agacaactga tctctactgt tatggagaat taaatgcacg tcaagggtg gaggatgaaa tagatggtcc 1600
1601 ggctggacaa gcagaaccgg acagggccca tacaatattg cttggaaatg tgaccttacg ctgcgacgtg gaattgatg 1680
1681 tctcaagcat ccgacaagta gaacaggtga ctttgaaaga ccttgaaaag ttgaccgcct gcgtgtgcaa gaaggtgtg 1760
1761 tctcaagcat CCGACTTCGG TACCAAGCTT AAGTTTAAAC CGCTGATCAG CCTCGACTGT GCCTTCTAGT TGCCAGCCAT 1840
1841 CTGTTGTTTG CCCCTCCCCC GTGCCTTCCT TGACCCTGGA AGGTGCCACT CCCACTGTCC TTTCCTAATA AAATGAGGAA 1920
1921 ATTGCATCGC ATTGTCTGAG TAGGTGTCAT TCTATTCTGG GGGGTGGGGT GGGGCAGGAC AGCAAGGGGG AGGATTGGGA 2000
2001 AGACAATAGC AGGCATGCTG GGGATGCGGT GGGCTCTATG GGTTCTAGAC TCGGGAAAGA ACCTGTCGTG CCAGCTGCAT 2080
2081 TAATGAATCG GCCAACGCGC GGGGAGAGGC GGTTTGCGTA TTGGGCGCTC TTCCGCTTCC TCGCTCACTG ACTCGCTGCG 2160
2161 CTCGGTCGTT CGGCTGCGGC GAGCGGTATC AGCTCACTCA AAGGCGGTAA TACGGTTATC CACAGAATCA GGGGATAACG 2240
2241 CAGGAAAGAA CATGTGAGCA AAAGGCCAGC AAAAGGCCAG GAACCGTAAA AAGGCCGCGT TGCTGGCGTT TTTCCATAGG 2320
2321 CTCCGCCCCC CTGACGAGCA TCACAAAAAT CGACGCTCAA GTCAGAGGTG GCGAAACCCG ACAGGACTAT AAAGATACCA 2400
2401 GGCGTTTCCC CCTGGAAGCT CCCTCGTGCG CTCTCCTGTT CCGACCCTGC CGCTTACCGG ATACCTGTCC GCCTTTCTCC 2480
2481 CTTCGGGAAG CGTGGCGCTT TCTCATAGCT CACGCTGTAG GTATCTCAGT TCGGTGTAGG TCGTTCGCTC CAAGCTGGGC 2560
2561 TGTGTGCACG AACCCCCCGT TCAGCCCGAC CGCTGCGCCT TATCCGGTAA CTATCGTCTT GAGTCCAACC CGGTAAGACA 2640
2641 CGACTTATCG CCACTGGCAG CAGCCACTGG TAACAGGATT AGCAGAGCGA GGTATGTAGG CGGTGCTACA GAGTTCTTGA 2720
2721 AGTGGTGGCC TAACTACGGC TACACTAGAA GGACAGTATT TGGTATCTGC GCTCTGCTGA AGCCAGTTAC CTTCGGAAAA 2800
2801 AGAGTTGGTA GCTCTTGATC CGGCAAACAA ACCACCGCTG GTAGCGGTGG TTTTTTTGTT TGCAAGCAGC AGATTACGCG 2880
2881 CAGAAAAAAA GGATCTCAAG AAGATCCTTT GATCTTTTCT ACGGGGTCTG ACGCTCAGTG GAACGAAAAC TCACGTTAAG 2960
2961 GGATTTTGGT CATGAGATTA TCAAAAAGGA TCTTCACCTA GATCCTTTTA AATTAAAAAT GAAGTTTTAA ATCAATCTAA 3040
3041 AGTATATATG AGTAAACTTG GTCTGACAGT TACCAATGCT TAATCAGTGA GGCACCTATC TCAGCGATCT GTCTATTTCG 3120
3121 TTCATCCATA GTTGCCTGAC TCCCCGTCGT GTAGATAACT ACGATACGGG AGGGCTTACC ATCTGGCCCC AGTGCTGCAA 3200
3201 TGATACCGCG AGACCCACGC TCACCGGCTC CAGATTTATC AGCAATAAAC CAGCCAGCCG GAAGGGCCGA GCGCAGAAGT 3280
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3281 CAATGCGGCG GCTGCATACG CTTGATCCGG CTACCTGCCC ATTCGACCAC CAAGCGAAAC ATCGCATCGA GCGAGCACGT 3360
3361 ACTCGGATGG AAGCCCGTCT TGTCGATCAG GATGATCTGG ACGAAGAGCA TCAGGGGCTC GCGCCAGCCG AACTGTTCGC 3440
3441 CAGGCTCAAG GCGCGCATGC CCGACGGCGA GGATCTCGTC GTGACCCATG GCGATGCCTG CTTGCCGAAT ATCATGGTGG 3520
3521 AAAATGGCCG CTTTTCTGGA TTCATCGACT GTGGCCGGCT GGGTGTGGCG GACCGCTATC AGGACATAGC GTTGGCTACC 3600
3601 CGTGATATTG CTGAAGAGCT TGGCGGCGAA TGGGCTGACC GCTTCCTCGT GCTTTACGGT ATCGCCGCTC CCGATTCGCA 3680
3681 GCGCATCGCC TTCTATCGCC TTCTTGACGA GTTCTTCTGA GCGGGACTCT GGGGTTCGAA ATGACCGACC AAGCGACGCC 3760
3761 CAACCTGCCA TCACGAGATT TCGATTCCAC CGCCGCCTTC TATGAAAGGT CGCCTTGTCG GCGTTTTTCC CGGGACGCCG 3840
3841 GCTGGATGAT CCTCCAGCGC GGGGATCTCA TGCTGGAGTT CTTCGCCCAC CCCAACTTGT TTATTGCAGC ATTACCTCTT 3920
3921 TACAAATAAA GCAATAGCAT CACAAATTTC ACAAATAAAG CATTTTTTTC ACTGCATTCT AGTTGTGGTT TGTCCAAACT 4000
4001 CATCAATGTA TCTTATCATG TCTGTATACC GTCGACCTCT AGCTAGAGCT TGGCGTAATC ATGGTCATAG CTGTTTCCTG 4080
4081 TGTGAAATTG TTATCCGCTC ACAATTCCAC ACAACATACG AGCCGGAAGC ATAAAGTGTA AAGCCTGGGG TGCCTAATGA 4160
4161 GTGAGCTAAC TCACATTAAT TGCGTTGCGC TCACTGCCCG CTTTCCAGTC GGGAAACCTG TCGTGCCAGC TGCATTAATG 4240
4241 AATCGGCCAA CGCGCGGGGA GAGGCGGTTT GCGTATTGGG CGCTCTTCCG CTTCCTCGCT CACTGACTCG CTGCGCTCGG 4320
4321 TCGTTCGGCT GCGGCGAGCG GTATCAGCTC ACTCAAAGGC GGTAATACGG TTATCCACAG AATCAGGGGA TAACGCAGGA 4400
4401 AAGAACATGT GAGCAAAAGG CCAGCAAAAG GCCAGGAACC GTAAAAAGGC CGCGTTGCTG GCGTTTTTCC ATAGGCTCCG 4480
4481 CCCCCCTGAC GAGCATCACA AAAATCGACG CTCAAGTCAG AGGTGGCGAA ACCCGACAGG ACTATAAAGA TACCAGGCGT 4560
4561 TTCCCCCTGG AAGCTCCCTC GTGCGCTCTC CTGTTCCGAC CCTGCCGCTT ACCGGATACC TGTCCGCCTT TCTCCCTTCG 4640
4641 GGAAGCGTGG CGCTTTCTCA ATGCTCACGC TGTAGGTATC TCAGTTCGGT GTAGGTCGTT CGCTCCAAGC TGGGCTGTGT 4720
4721 GCACGAACCC CCCGTTCAGC CCGACCGCTG CGCCTTATCC GGTAACTATC GTCTTGAGTC CAACCCGGTA AGACACGACT 4800
4801 TATCGCCACT GGCAGCAGCC ACTGGTAACA GGATTAGCAG AGCGAGGTAT GTAGGCGGTG CTACAGAGTT CTTGAAGTGG 4880
4881 TGGCCTAACT ACGGCTACAC TAGAAGGACA GTATTTGGTA TCTGCGCTCT GCTGAAGCCA GTTACCTTCG GAAAAAGAGT 4960
4961 TGGTAGCTCT TGATCCGGCA AACAAACCAC CGCTGGTAGC GGTGGTTTTT TTGTTTGCAA GCAGCAGATT ACGCGCAGAA 5040
5041 AAAAAGGATC TCAAGAAGAT CCTTTGATCT TTTCTACGGG GTCTGACGCT CAGTGGAACG AAAACTCACG TTAAGGGATT 5120
5121 TTGGTCATGA GATTATCAAA AAGGATCTTC ACCTAGATCC TTTTAAATTA AAAATGAAGT TTTAAATCAA TCTAAAGTAT 5200
5201 ATATGAGTAA ACTTGGTCTG ACAGTTACCA ATGCTTAATC AGTGAGGCAC CTATCTCAGC GATCTGTCTA TTTCGTTCAT 5280
5281 CCATAGTTGC CTGACTCCCC GTCGTGTAGA TAACTACGAT ACGGGAGGGC TTACCATCTG GCCCCAGTGC TGCAATGATA 5360
5361 CCGCGAGACC CACGCTCACC GGCTCCAGAT TTATCAGCAA TAAACCAGCC AGCCGGAAGG GCCGAGCGCA GAAGTGGTCC 5440
5441 TGCAACTTTA TCCGCCTCCA TCCAGTCTAT TAATTGTTGC CGGGAAGCTA GAGTAAGTAG TTCGCCAGTT AATAGTTTGC 5520
5521 GCAACGTTGT TGCCATTGCT ACAGGCATCG TGGTGTCACG CTCGTCGTTT GGTATGGCTT CATTCAGCTC CGGTTCCCAA 5600
5601 CGATCAAGGC GAGTTACATG ATCCCCCATG TTGTGCAAAA AAGCGGTTAG CTCCTTCGGT CCTCCGATCG TTGTCAGAAG 5680
5681 TAAGTTGGCC GCAGTGTTAT CACTCATGGT TATGGCAGCA CTGCATAATT CTCTTACTGT CATGCCATCC GTAAGATGCT 5760
5761 TTTCTGTGAC TGGTGAGTAC TCAACCAAGT CATTCTGAGA ATAGTGTATG CGGCGACCGA GTTGCTCTTG CCCGGCGTCA 5840
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5841 ATACGGGATA ATACCGCGCC ACATAGCAGA ACTTTAAAAG TGCTCATCAT TGGAAAACGT TCTTCGGGGC GAAAACTCTC 5920
6001 CAAGCTGGCT GTGTGCACGA ACCCCCCGTT CAGCCCGACC GCTGCGCCTT ATCCGGTAAC TATCGTCTTG AGTCCAACCC 6000
6081 CTCATACTCT TCCTTTTTCA ATATTATTGA AGCATTTATC AGGGTTATTG TCTCATGAGC GGATACATAT TTGAATGTAT 6160
6161 TTAGAAAAAT AAACAAATAG GGGTTCCGCG CACATTTCCC CGAAAAGTGC CACCTGACGT C 70 80 6221
      10 20 30 40 50 60
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GENERAL RECOMBINANT DNA METHODS

Basic texts disclosing general methods of molecular biology, all of which are incorporated by reference, include: Sambrook, J *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989; Ausubel, FM *et al. Current Protocols in Molecular Biology*, Vol. 2, Wiley-Interscience, New York, (current edition); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); Glover, DM, ed, *DNA Cloning: A Practical Approach*, vol. I & II, IRL Press, 1985; Albers, B. *et al.*, *Molecular Biology of the Cell*, 2nd Ed., Garland Publishing, Inc., New York, NY (1989); Watson, JD *et al.*, *Recombinant DNA*, 2nd Ed., Scientific American Books, New York, 1992; and Old, RW *et al.*, *Principles of Gene Manipulation: An Introduction to Genetic Engineering*, 2nd Ed., University of California Press, Berkeley, CA (1981).

Unless otherwise indicated, a particular nucleic acid sequence is intended to encompasses conservative substitution variants thereof *(e.g.,* degenerate codon substitutions) and a complementary sequence. The term "nucleic acid" is synonymous with "polynucleotide" and is intended to include a gene, a cDNA molecule, an mRNA molecule, as well as a fragment of any of these such as an oligonucleotide, and further, equivalents thereof (explained more fully below). Sizes of nucleic acids are stated either as kilobases (kb) or base pairs (bp). These are estimates derived from agarose or polyacrylamide gel electrophoresis (PAGE), from nucleic acid sequences which are determined by the user or published. Protein size is stated as molecular mass in kilodaltons (kDa) or as length (number of amino acid residues). Protein size is estimated from PAGE, from sequencing, from presumptive amino acid sequences based on the coding nucleic acid sequence or from published amino acid sequences.

Specifically, cDNA molecules encoding the amino acid sequence corresponding to the fusion polypeptide of the present invention or fragments or derivatives thereof can be synthesized by the polymerase chain reaction (PCR) (see, for example, U.S. 4,683,202) using primers derived the sequence of the protein disclosed herein. These cDNA sequences can then be assembled into a eukaryotic or prokaryotic expression vector and the resulting vector can be used to direct the synthesis of the fusion polypeptide or its fragment or derivative by appropriate host cells, for example COS or CHO cells.

This invention includes isolated nucleic acids having a nucleotide sequence encoding the novel fusion polypeptides that comprise a translocation polypeptide and an antigen, fragments

thereof or equivalents thereof. The term nucleic acid as used herein is intended to include such fragments or equivalents. The nucleic acid sequences of this invention can be DNA or RNA.

A cDNA nucleotide sequence the fusion polypeptide can be obtained by isolating total mRNA from an appropriate cell line. Double stranded cDNA is prepared from total mRNA. cDNA can be inserted into a suitable plasmid, bacteriophage or viral vector using any one of a number of known techniques.

In reference to a nucleotide sequence, the term "equivalent" is intended to include sequences encoding structurally homologous and/or a functionally equivalent proteins. For example, a natural polymorphism in ETA(dII) nucleotide sequence (especially at the third base of a codon) may be manifest as "silent" mutations which do not change the amino acid sequence. Furthermore, there may be one or more naturally occurring isoforms or related, immunologically cross-reactive family members of these proteins. Such isoforms or family members are defined as proteins that share function amino acid sequence similarity to, for example, ETA(dII)

Fragment of Nucleic Acid

A fragment of the nucleic acid sequence is defined as a nucleotide sequence having fewer nucleotides than the nucleotide sequence encoding the full length translocation polypeptide, antigenic polypeptide or the fusion thereof.. This invention includes such nucleic acid fragments that encode polypeptides which retain (1) the ability of the fusion polypeptide to induce increases in frequency or reactivity of T cells, preferably CD8+ T cells, that are specific for the antigen part of the fusion polypeptide.

For example, a nucleic acid fragment as intended herein encodes a ETA(dII) polypeptide that retains the ability to improve the immunogenicity of an antigen when administered as a fusion polypeptide with an antigenic polypeptide or peptide.

Generally, the nucleic acid sequence encoding a fragment of a ETA(dII) polypeptide comprises of nucleotides from the sequence encoding the mature protein (or an active fragment thereof).

Nucleic acid sequences of this invention may also include linker sequences, natural or modified restriction endonuclease sites and other sequences that are useful for manipulations related to cloning, expression or purification of encoded protein or fragments. These and other modifications of nucleic acid sequences are described herein or are well-known in the art.

The techniques for assembling and expressing DNA coding sequences for translocation types of proteins, and DNA coding sequences for antigenic polypeptides, include synthesis of oligonucleotides, PCR, transforming cells, constructing vectors, expression systems, and the like; these are well-established in the art such that those of ordinary skill are familiar with standard resource materials, specific conditions and procedures.

EXPRESSION VECTORS AND HOST CELLS

This invention includes an expression vector comprising a nucleic acid sequence encoding a translocation polypeptide/antigen fusion polypeptide, preferably a ETA(dII)/antigen fusion polypeptide operably linked to at least one regulatory sequence.

The term "expression vector" or "expression cassette" as used herein refers to a nucleotide sequence which is capable of affecting expression of a protein coding sequence in a host compatible with such sequences. Expression cassettes include at least a promoter operably linked with the polypeptide coding sequence; and, optionally, with other sequences, *e.g.*, transcription termination signals. Additional factors necessary or helpful in effecting expression may also be included, *e.g.*, enhancers.

"Operably linked" means that the coding sequence is linked to a regulatory sequence in a manner that allows expression of the coding sequence. Known regulatory sequences are selected to direct expression of the desired protein in an appropriate host cell. Accordingly, the term "regulatory sequence" includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in, for example, Goeddel, *Gene Expression Technology. Methods in Enzymology*, vol. 185, Academic Press, San Diego, Calif. (1990)).

Thus, expression cassettes include plasmids, recombinant viruses, any form of a recombinant "naked DNA" vector, and the like. A "vector" comprises a nucleic acid which can infect, transfect, transiently or permanently transduce a cell. It will be recognized that a vector can be a naked nucleic acid, or a nucleic acid complexed with protein or lipid. The vector optionally comprises viral or bacterial nucleic acids and/or proteins, and/or membranes (*e.g.*, a cell membrane, a viral lipid envelope, *etc.*). Vectors include, but are not limited to replicons (*e.g.*, RNA replicons (see Example 1, below), bacteriophages) to which fragments of DNA may be attached and become replicated. Vectors thus include, but are not limited to RNA, autonomous self-replicating circular or linear DNA or RNA, *e.g.*, plasmids, viruses, and the like (U.S. Patent No. 5,217,879), and includes both the expression and nonexpression plasmids.

Where a recombinant microorganism or cell culture is described as hosting an "expression vector" this includes both extrachromosomal circular and linear DNA and DNA that has been incorporated into the host chromosome(s). Where a vector is being maintained by a host cell, the vector may either be stably replicated by the cells during mitosis as an autonomous structure, or is incorporated within the host's genome.

Those skilled in the art appreciate that the particular design of an expression vector of this invention depends on considerations such as the host cell to be transfected and/or the type of protein to be expressed.

The present expression vectors comprise the full range of nucleic acid molecules encoding the various embodiments of the fusion polypeptide and its functional derivatives (defined herein) including polypeptide fragments, variants, *etc.*

Such expression vectors are used to transfect host cells (*in vitro*, *ex vivo* or *in vivo*) for expression of the DNA and production of the encoded proteins which include fusion proteins or peptides. It will be understood that a genetically modified cell expressing the fusion polypeptide may transiently express the exogenous DNA for a time sufficient for the cell to be useful for its stated purpose.

The present in invention provides methods for producing the fusion polypeptides, fragments and derivatives. For example, a host cell transfected with a nucleic acid vector that encodes the fusion polypeptide is cultured under appropriate conditions to allow expression of the polypeptide.

Host cells may also be transfected with one or more expression vectors that singly or in combination comprise DNA encoding at least a portion of the fusion polypeptide and DNA encoding at least a portion of a second protein, so that the host cells produce yet further fusion polypeptides that include both the portions.

A culture typically includes host cells, appropriate growth media and other byproducts. Suitable culture media are well known in the art. The fusion polypeptide can be isolated from medium or cell lysates using conventional techniques for purifying proteins and peptides, including ammonium sulfate precipitation, fractionation column chromatography (e.g. ion exchange, gel filtration, affinity chromatography, etc.) and/or electrophoresis (see generally, "Enzyme Purification and Related Techniques", *Methods in Enzymology*, 22:233-577 (1971)).

Once purified, partially or to homogeneity, the recombinant polypeptides of the invention can be utilized in pharmaceutical compositions as described in more detail herein.

The term "isolated" as used herein, when referring to a molecule or composition, such as a translocation polypeptide or a nucleic acid coding therefor, means that the molecule or composition is separated from at least one other compound (protein, other nucleic acid, *etc.*) or from other contaminants with which it is natively associated or becomes associated during processing.. An isolated composition can also be substantially pure. An isolated composition can be in a homogeneous state and can be dry or in aqueous solution. Purity and homogeneity can be determined, for example, using analytical chemical techniques such as polyacrylamide gel electrophoresis (PAGE) or high performance liquid chromatography (HPLC). Even where a protein has been isolated so as to appear as a homogenous or dominant band in a gel pattern, there are trace contaminants which co-purify with it.

Prokaryotic or eukaryotic host cells transformed or transfected to express the fusion polypeptide or a homologue or functional derivative thereof are within the scope of the invention. For example, the fusion polypeptide may be expressed in bacterial cells such as *E. coli*, insect cells (baculovirus), yeast, or mammalian cells such as Chinese hamster ovary cells (CHO) or human cells. Other suitable host cells may be found in Goeddel, (1990) *supra* or are otherwise known to those skilled in the art.

Expression in eukaryotic cells leads to partial or complete glycosylation and/or formation of relevant inter- or intra-chain disulfide bonds of the recombinant protein.

Although preferred vectors are described in the Examples, other examples of expression vectors are provided here. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari *et al.*, (1987) *EMBO J.* 6:229-234), pMFa (Kurjan *et al.* (1982) *Cell* 30:933-943), pJRY88 (Schultz *et al.*, (1987) *Gene* 54:113-123), and pYES2 (Invitrogen Corporation, San Diego, Calif.). Baculovirus vectors available for expression of proteins in cultured insect cells (SF 9 cells) include the pAc series (Smith *et al.*, (1983) *Mol. Cell Biol.* 3:2156-2165,) and the pVL series (Lucklow, V. A., and Summers, M. D., (1989) *Virology* 170:31-39). Generally, COS cells (Gluzman, Y., (1981) *Cell* 23:175-182) are used in conjunction with such vectors as pCDM 8 (Aruffo A. and Seed, B., *supra,* for transient amplification/expression in mammalian cells, while CHO (*dhfr*-negative CHO) cells are used with vectors such as pMT2PC (Kaufman *et al.* (1987), *EMBO J.* 6:187-195) for stable amplification/expression in mammalian cells. The

NS0 myeloma cell line (a glutamine synthetase expression system.) is available from Celltech Ltd.

Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the reporter group and the target protein to enable separation of the target protein from the reporter group subsequent to purification of the fusion protein. Proteolytic enzymes for such cleavage and their recognition sequences include Factor Xa, thrombin and enterokinase.

Typical fusion expression vectors include pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase, maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Inducible non-fusion expression vectors include pTrc (Amann *et al.*, (1988) *Gene* 69:301-315) and pET 11d (Studier *et al.*, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, Calif. (1990) 60-89). While target gene expression relies on host RNA polymerase transcription from the hybrid trp-lac fusion promoter in pTrc, expression of target genes inserted into pET 11d relies on transcription from the T7 gn10-lacO fusion promoter mediated by coexpressed viral RNA polymerase (T7gn1). Th is viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident λ prophage harboring a T7gn1 under the transcriptional control of the lacUV 5 promoter.

One embodiment of this invention is a transfected cell which expresses novel fusion polypeptide.

Vector Construction

Construction of suitable vectors containing the desired coding and control sequences employs standard ligation and restriction techniques which are well understood in the art. Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and re-ligated in the form desired.

The DNA sequences which form the vectors are available from a number of sources. Backbone vectors and control systems are generally found on available "host" vectors which are used for the bulk of the sequences in construction. For the pertinent coding sequence, initial construction may be, and usually is, a matter of retrieving the appropriate sequences from cDNA or genomic DNA libraries. However, once the sequence is disclosed it is possible to synthesize the entire gene sequence *in vitro* starting from the individual nucleotide derivatives. The entire

gene sequence for genes of sizeable length, *e.g.*, 500-1000 bp may be prepared by synthesizing individual overlapping complementary oligonucleotides and filling in single stranded nonoverlapping portions using DNA polymerase in the presence of the deoxyribonucleotide triphosphates. This approach has been used successfully in the construction of several genes of known sequence. See, for example, Edge, M. D., *Nature* (1981) 292:756; Nambair, K. P., *et al.*, *Science* (1984) 223:1299; and Jay, E., *J Biol Chem* (1984) 259:6311.

Synthetic oligonucleotides are prepared by either the phosphotriester method as described by references cited above or the phosphoramidite method as described by Beaucage, S. L., and Caruthers, M. H., *Tet Lett* (1981) 22:1859; and Matteucci, M. D., and Caruthers, M. H., *J Am Chem Soc* (1981) 103:3185 and can be prepared using commercially available automated oligonucleotide synthesizers. Kinase treatment of single strands prior to annealing or for labeling is achieved using an excess, e.g., about 10 units of polynucleotide kinase to 1 nmole substrate in the presence of 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1-2 mM ATP, 1.7 pmoles γ-^{32}P-ATP (2.9 mCi/mmole), 0.1 mM spermidine, 0.1 mM EDTA.

Once the components of the desired vectors are thus available, they can be excised and ligated using standard restriction and ligation procedures. Site-specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, *e.g.*, New England Biolabs, Product Catalog. In general, about 1 mg of plasmid or DNA sequence is cleaved by one unit of enzyme in about 20 ml of buffer solution; in the examples herein, typically, an excess of restriction enzyme is used to insure complete digestion of the DNA substrate. Incubation times of about one hour to two hours at about 37°C. are workable, although variations can be tolerated. After each incubation, protein is removed by extraction with phenol/chloroform, and may be followed by ether extraction, and the nucleic acid recovered from aqueous fractions by precipitation with ethanol. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in *Methods in Enzymology* (1980) 65:499-560.

Restriction cleaved fragments may be blunt ended by treating with the large fragment of *E. coli* DNA polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTPs) using conventional methods and conditions. Ligations are performed using known,

conventional methods. In vector construction employing "vector fragments", the fragment is commonly treated with bacterial alkaline phosphatase (BAP) or calf intestinal alkaline phosphatase (CIAP) in order to remove the 5' phosphate and prevent self- Alternatively, re-ligation can be prevented in vectors which have been double digested by additional restriction enzyme and separation of the unwanted fragments.

Any of a number of methods are used to introduce mutations into the coding sequence to generate the variants of the invention. These mutations include simple deletions or insertions, systematic deletions, insertions or substitutions of clusters of bases or substitutions of single bases.

For example, modifications of the ETA(dII) polypeptide or the antigenic polypeptide DNA sequence are created by site-directed mutagenesis, a well-known technique for which protocols and reagents are commercially available (Zoller, MJ *et al., Nucleic Acids Res* (1982) 10:6487-6500 and Adelman, JP *et al., DNA* (1983) 2:183-193)). Correct ligations for plasmid construction are confirmed, for example, by first transforming *E. coli* strain MC1061 (Casadaban, M., *et al., J Mol Biol* (1980) 138:179-207) or other suitable host with the ligation mixture. Using conventional methods, transformants are selected based on the presence of the ampicillin-, tetracycline- or other antibiotic resistance gene (or other selectable marker) depending on the mode of plasmid construction. Plasmids are then prepared from the transformants with optional chloramphenicol amplification optionally following chloramphenicol amplification ((Clewell, DB *et al.* , *Proc Natl Acad Sci USA* (1969) 62:1159; Clewell, D. B., *J Bacteriol* (1972) 110:667). Several mini DNA preps are commonly used. See, *e.g.*,, Holmes, DS, *et al., Anal Biochem* (1981) 114:193-197; Birnboim, HC *et al.* , *Nucleic Acids Res* (1979) 7:1513-1523. The isolated DNA is analyzed by restriction and/or sequenced by the dideoxy nucleotide method of Sanger (*Proc Natl Acad Sci USA* (1977) 74:5463) as further described by Messing, *et al., Nucleic Acids Res* (1981) 9:309, or by the method of Maxam *et al. Methods in Enzymology* (1980) 65:499.

Vector DNA can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming host cells can be found in Sambrook *et al. supra* and other standard texts.

Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the reporter group and the target protein to enable separation of the target protein from the reporter group subsequent to purification of the fusion protein. Proteolytic enzymes for such cleavage and their recognition sequences include Factor Xa, thrombin and enterokinase.

Known fusion expression vectors include pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase, maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Inducible non-fusion expression vectors include pTrc (Amann *et al.*, (1988) *Gene* 69:301-315) and pET 11d (Studier *et al.*, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, Calif. (1990) 60-89). While target gene expression relies on host RNA polymerase transcription from the hybrid trp-lac fusion promoter in pTrc, expression of target genes inserted into pET 11d relies on transcription from the T7 gn10-lacO fusion promoter mediated by coexpressed viral RNA polymerase (T7gn1). Th is viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident λ prophage harboring a T7gn1 under the transcriptional control of the lacUV 5 promoter.

Promoters and Enhancers

A promoter region of a DNA or RNA molecule binds RNA polymerase and promotes the transcription of an "operably linked" nucleic acid sequence. As used herein, a "promoter sequence" is the nucleotide sequence of the promoter which is found on that strand of the DNA or RNA which is transcribed by the RNA polymerase. Two sequences of a nucleic acid molecule, such as a promoter and a coding sequence, are "operably linked" when they are linked to each other in a manner which permits both sequences to be transcribed onto the same RNA transcript or permits an RNA transcript begun in one sequence to be extended into the second sequence. Thus, two sequences, such as a promoter sequence and a coding sequence of DNA or RNA are operably linked if transcription commencing in the promoter sequence will produce an RNA transcript of the operably linked coding sequence. In order to be "operably linked" it is not necessary that two sequences be immediately adjacent to one another in the linear sequence.

The preferred promoter sequences of the present invention must be operable in mammalian cells and may be either eukaryotic or viral promoters. Although preferred promoters are described in the Examples, other useful promoters and regulatory elements are discussed

below. Suitable promoters may be inducible, repressible or constitutive. A "constitutive" promoter is one which is active under most conditions encountered in the cell's environmental and throughout development. An "inducible" promoter is one which is under environmental or developmental regulation. A "tissue specific" promoter is active in certain tissue types of an organism. An example of a constitutive promoter is the viral promoter MSV-LTR, which is efficient and active in a variety of cell types, and, in contrast to most other promoters, has the same enhancing activity in arrested and growing cells. Other preferred viral promoters include that present in the CMV-LTR (from cytomegalovirus) (Bashart, M. *et al.*, *Cell 41*:521 (1985)) or in the RSV-LTR (from Rous sarcoma virus) (Gorman, C.M., *Proc. Natl. Acad. Sci. USA 79*:6777 (1982). Also useful are the promoter of the mouse metallothionein I gene (Hamer, D., *et al.*, *J. Mol. Appl. Gen. 1*:273-288 (1982)); the TK promoter of Herpes virus (McKnight, S., *Cell 31*:355-365 (1982)); the SV40 early promoter (Benoist, C., *et al.*, *Nature 290*:304-310 (1981)); and the yeast *gal4* gene promoter (Johnston, S.A., *et al.*, *Proc. Natl. Acad. Sci. (USA) 79*:6971-6975 (1982); Silver, P.A., *et al.*, *Proc. Natl. Acad. Sci. (USA) 81*:5951-5955 (1984)). Other illustrative descriptions of transcriptional factor association with promoter regions and the separate activation and DNA binding of transcription factors include: Keegan *et al.*, *Nature* (1986) *231*:699; Fields *et al.*, *Nature* (1989) 340:245; Jones, *Cell* (1990) *61*:9; Lewin, Cell (1990) *61*:1161; Ptashne *et al.*, *Nature* (1990) *346*:329; Adams *et al.*, *Cell* (1993) 72:306. The relevant disclosure of all of these above-listed references is hereby incorporated by reference.

The promoter region may further include an octamer region which may also function as a tissue specific enhancer, by interacting with certain proteins found in the specific tissue. The enhancer domain of the DNA construct of the present invention is one which is specific for the target cells to be transfected, or is highly activated by cellular factors of such target cells. Examples of vectors (plasmid or retrovirus) are disclosed in (Roy-Burman *et al.*, U.S. Patent No. 5,112,767). For a general discussion of enhancers and their actions in transcription, see, Lewin, B.M., *Genes IV*, Oxford University Press, Oxford, (1990), pp. 552-576. Particularly useful are retroviral enhancers (*e.g.*, viral LTR). The enhancer is preferably placed upstream from the promoter with which it interacts to stimulate gene expression. For use with retroviral vectors, the endogenous viral LTR may be rendered enhancer-less and substituted with other desired enhancer sequences which confer tissue specificity or other desirable properties such as transcriptional efficiency.

Nucleic acids of the invention can also be chemically synthesized using standard techniques. Various methods of chemically synthesizing polydeoxynucleotides are known, including solid-phase synthesis which, like peptide synthesis, has been fully automated with commercially available DNA synthesizers (See, *e.g.*, Itakura *et al.* U.S. Pat. No. 4,598,049; Caruthers *et al.* U.S. Pat. No. 4,458,066; and Itakura U.S. Pat. Nos. 4,401,796 and 4,373,071, incorporated by reference herein).

PROTEINS AND POLYPEPTIDES

The terms "polypeptide," "protein," and "peptide" when referring to compositions of the invention are meant to include variants, analogues, and mimetics with structures and/or activity that substantially correspond to the polypeptide or peptide from which the variant, *etc.*, was derived.

The present invention includes an "isolated" fusion polypeptide comprising a translocation polypeptide linked to an antigenic polypeptide. A preferred translocation polypeptide is ETA(dII), preferably SEQ ID NO:3. A preferred fusion polypeptide is ETA(dII)/E7, *e.g.*, residues 1-269 of SEQ ID NO:5. While the present disclosure exemplifies a particular ETA(dII) sequence, it is to be understood that homologues of ETA(dII) from other bacteria (or from eukaryotic origin if such are found) and mutants thereof that possess the characteristics disclosed herein are intended within the scope of this invention.

The term "chimeric" or "fusion" polypeptide or protein refers to a composition comprising at least one polypeptide or peptide sequence or domain that is chemically bound in a linear fashion with a second polypeptide or peptide domain. One embodiment of this invention is an isolated or recombinant nucleic acid molecule encoding a fusion protein comprising at least two domains, wherein the first domain comprises a translocation polypeptide, *e.g.*, ETA(dII), and the second domain comprising an antigenic epitope, *e.g.*, an MHC class I-binding peptide epitope. Additional domains can comprise a polypeptide, peptide, polysaccharide, or the like. The "fusion" can be an association generated by a peptide bond, a chemical linking, a charge interaction (*e.g.*, electrostatic attractions, such as salt bridges, H-bonding, *etc.*) or the like. If the polypeptides are recombinant, the "fusion protein" can be translated from a common mRNA. Alternatively, the compositions of the domains can be linked by any chemical or electrostatic means. The chimeric molecules of the invention (*e.g.*, translocation polypeptide fusion proteins) can also include additional sequences, *e.g.*, linkers, epitope tags, enzyme cleavage recognition

sequences, signal sequences, secretion signals, and the like. Alternatively, a peptide can be linked to a carrier simply to facilitate manipulation or identification/ location of the peptide.

Also included is a "functional derivative" of ETA(dII), which refers to an amino acid substitution variant, a "fragment," or a "chemical derivative" of the protein, which terms are defined below. A functional derivative retains measurable ETA(dII)-like activity, preferably that of promoting immunogenicity of one or more antigenic epitopes fused thereto by, *e.g.*, promoting presentation by class I pathways which permits the "functional derivative's" utility in accordance with the present invention. "Functional derivatives" encompass "variants" and "fragments" regardless of whether the terms are used in the conjunctive or the alternative herein.

A functional homologue must possess the above biochemical and biological activity. In view of this functional characterization, use of homologous ETA(dII) proteins including proteins not yet discovered, fall within the scope of the invention if these proteins have sequence similarity and the recited biochemical and biological activity.

To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred method of alignment, Cys residues are aligned.

In a preferred embodiment, the length of a sequence being compared is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence (*e.g.*, ETA(dII), SEQ ID NO:3). The amino acid residues (or nucleotides) at corresponding amino acid (or nucleotide) positions are then compared. When a position in the first sequence is occupied by the same amino acid residue (or nucleotide) as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the

percent identity between two amino acid sequences is determined using the Needleman and Wunsch (*J. Mol. Biol. 48*:444-453 (1970) algorithm which has been incorporated into the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Meyers and W. Miller (CABIOS, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases, for example, to identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul *et al.* (1990) *J. Mol. Biol. 215*:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to ETA(dII) nucleic acid molecules. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to HVP22 protein molecules. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res. 25*:3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

Thus, a homologue of ETA(dII) as described above is characterized as having (a) functional activity of native ETA(dII) and (b) sequence similarity to a native ETA(dII) protein (such as SEQ ID NO:3) when determined as above, of at least about 20% (at the amino acid level), preferably at least about 40%, more preferably at least about 70%, even more preferably at least about 90%.

It is within the skill in the art to obtain and express such a protein using DNA probes based on the disclosed sequences of ETA(dII). Then, the fusion protein's biochemical and

biological activity can be tested readily using art-recognized methods such as those described herein, for example, a T cell proliferation, cytokine secretion or a cytolytic assay, or an *in vivo* assay of tumor protection or tumor therapy. A biological assay of the stimulation of antigen-specific T cell reactivity will indicate whether the homologue has the requisite activity to qualify as a "functional" homologue.

A "variant" refers to a molecule substantially identical to either the full protein or to a fragment thereof in which one or more amino acid residues have been replaced (substitution variant) or which has one or several residues deleted (deletion variant) or added (addition variant). A "fragment" of ETA(dII) refers to any subset of the molecule, that is, a shorter polypeptide of the full-length protein.

A number of processes can be used to generate fragments, mutants and variants of the isolated DNA sequence. Small subregions or fragments of the nucleic acid encoding the spreading protein, for example 1-30 bases in length, can be prepared by standard, chemical synthesis. Antisense oligonucleotides and primers for use in the generation of larger synthetic fragment.

A preferred group of variants are those in which at least one amino acid residue and preferably, only one, has been substituted by different residue. For a detailed description of protein chemistry and structure, see Schulz, GE *et al.*, *Principles of Protein Structure*, Springer-Verlag, New York, 1978, and Creighton, T.E., *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, 1983, which are hereby incorporated by reference. The types of substitutions that may be made in the protein molecule may be based on analysis of the frequencies of amino acid changes between a homologous protein of different species, such as those presented in Table 1-2 of Schulz *et al. (supra)* and Figure 3-9 of Creighton (*supra*). Based on such an analysis, conservative substitutions are defined herein as exchanges within one of the following five groups:

1	Small aliphatic, nonpolar or slightly polar residues	Ala, Ser, Thr (Pro, Gly);
2	Polar, negatively charged residues and their amides	Asp, Asn, Glu, Gln;
3	Polar, positively charged residues	His, Arg, Lys;
4	Large aliphatic, nonpolar residues	Met, Leu, Ile, Val (Cys)
5	Large aromatic residues	Phe, Tyr, Trp.

The three amino acid residues in parentheses above have special roles in protein architecture. Gly is the only residue lacking a side chain and thus imparts flexibility to the chain. Pro, because of its unusual geometry, tightly constrains the chain. Cys can participate in disulfide bond formation, which is important in protein folding.

Morc substantial changes in biochemical, functional (or immunological) properties are made by selecting substitutions that are less conservative, such as between, rather than within, the above five groups. Such changes will differ more significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Examples of such substitutions are (i) substitution of Gly and/or Pro by another amino acid or deletion or insertion of Gly or Pro; (ii) substitution of a hydrophilic residue, *e.g.*, Ser or Thr, for (or by) a hydrophobic residue, *e.g.*,, Leu, Ile, Phe, Val or Ala; (iii) substitution of a Cys residue for (or by) any other residue; (iv) substitution of a residue having an electropositive side chain, *e.g.*,, Lys, Arg or His, for (or by) a residue having an electronegative charge, *e.g.*,, Glu or Asp; or (v) substitution of a residue having a bulky side chain, *e.g.*, Phe, for (or by) a residue not having such a side chain, *e.g.*, Gly.

Most acceptable deletions, insertions and substitutions according to the present invention are those that do not produce radical changes in the characteristics of the wild-type or native protein in terms of its intercellular spreading activity and its ability to stimulate antigen specific T cell reactivity to an antigenic epitope or epitopes that are fused to the spreading protein. However, when it is difficult to predict the exact effect of the substitution, deletion or insertion in advance of doing so, one skilled in the art will appreciate that the effect can be evaluated by routine screening assays such as those described here, without requiring undue experimentation.

Whereas shorter chain variants can be made by chemical synthesis, for the present invention, the preferred longer chain variants are typically made by site-specific mutagenesis of the nucleic acid encoding the polypeptide, expression of the variant nucleic acid in cell culture, and, optionally, purification of the polypeptide from the cell culture, for example, by immunoaffinity chromatography using specific antibody immobilized to a column (to absorb the variant by binding to at least one epitope).

The term "chemically linked" refers to any chemical bonding of two moieties, *e.g.*, as in one embodiment of the invention, where a translocation polypeptide is chemically linked to an

antigenic peptide. Such chemical linking includes the peptide bonds of a recombinantly or *in vivo* generated fusion protein.

Chemical Derivatives

"Chemical derivatives" of the polypeptide or fusion polypeptide of the invention contain additional chemical moieties not normally a part of the protein. Covalent modifications of the polypeptide are included within the scope of this invention. Such derivatized moieties may improve the solubility, absorption, biological half life, and the like. Moieties capable of mediating such effects are disclosed, for example, in *Remington's Pharmaceutical Sciences*, 16th ed., Mack Publishing Co., Easton, PA (1980).

Such modifications may be introduced into the molecule by reacting targeted amino acid residues of the polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues. Another modification is cyclization of the protein. Examples of chemical derivatives of the polypeptide follow.

Lysinyl and amino terminal residues are derivatized with succinic or other carboxylic acid anhydrides. Derivatization with a cyclic carboxylic anhydride has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Carboxyl side groups, aspartyl or glutamyl, may be selectively modified by reaction with carbodiimides (R-N=C=N-R') such as 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues can be converted to asparaginyl and glutaminyl residues by reaction with ammonia.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the amino group of lysine (Creighton, *supra*, pp. 79-86), acetylation of the N-terminal amine, and amidation of the C-terminal carboxyl groups.

Also included are peptides wherein one or more D-amino acids are substituted for one or more L-amino acids.

Multimeric Peptides

The present invention also includes longer polypeptides in which a basic peptidic sequence obtained from the sequence of a translocation polypeptide, or the antigenic polypeptide or peptide unit, is repeated from about two to about 100 times, with or without intervening spacers or linkers. It is understood that such multimers may be built from any of the peptide variants defined herein. Moreover, a peptide multimer may comprise different combinations of peptide monomers and the disclosed substitution variants thereof. Such oligomeric or multimeric peptides can be made by chemical synthesis or by recombinant DNA techniques as discussed herein. When produced chemically, the oligomers preferably have from 2-12 repeats of the basic peptide sequence. When produced recombinantly, the multimers may have as many repeats as the expression system permits, for example from two to about 100 repeats.

In tandem multimers, preferably dimers and trimers, of the fusion polypeptide, the chains bonded by interchain disulfide bonds or other "artificial" covalent bonds between the chains such that the chains are "side-by-side" rather than "end to end."

THERAPEUTIC COMPOSITIONS AND THEIR ADMINISTRATION

A vaccine composition comprising the nucleic acid encoding the fusion polypeptide, or a cell expressing this nucleic acid is administered to a mammalian subject, preferably a human. The vaccine composition is administered in a pharmaceutically acceptable carrier in a biologically effective or a therapeutically effective amount . The composition may be given alone or in combination with another protein or peptide such as an immunostimulatory molecule. Treatment may include administration of an adjuvant, used in its broadest sense to include any nonspecific immune stimulating compound such as an interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether.

A therapeutically effective amount is a dosage that, when given for an effective period of time, achieves the desired immunological or clinical effect.

A therapeutically active amount of a nucleic acid encoding the fusion polypeptide may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the peptide to elicit a desired response in the individual. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies

of the therapeutic situation. A therapeutically effective amounts of the protein, in cell associated form may be stated in terms of the protein or cell equivalents.

Thus an effective amount is between about 1 nanogram and about 1 gram per kilogram of body weight of the recipient, more preferably between about 0.1 μg/kg and about 10mg/kg, more preferably between about 1 μg/kg and about 1 mg/kg. Dosage forms suitable for internal administration preferably contain (for the latter dose range) from about 0.1 μg to 100 μg of active ingredient per unit. The active ingredient may vary from 0.5 to 95% by weight based on the total weight of the composition. Alternatively, an effective dose of cells expressing the nucleic acid is between about 10^4 and 10^8 cells. Those skilled in the art of immunotherapy will be able to adjust these doses without undue experimentation.

The active compound may be administered in a convenient manner, *e.g.*, injection by a convenient and effective route. Preferred routes include subcutaneous, intradermal, intravenous and intramuscular routes. Other possible routes include oral administration, intrathecal, inhalation, transdermal application, or rectal administration. For the treatment of existing tumors which have not been completely resected or which have recurred, direct intratumoral injection is also intended.

Depending on the route of administration, the active compound may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound. Thus it may be necessary to coat the composition with, or co-administer the composition with, a material to prevent its inactivation. For example, an enzyme inhibitors of nucleases or proteases (*e.g.*, pancreatic trypsin inhibitor, diisopropylfluorophosphate and trasylol).or in an appropriate carrier such as liposomes (including water-in-oil-in-water emulsions as well as conventional liposomes (Strejan *et al.*, (1984) *J. Neuroimmunol* 7:27).

As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the therapeutic compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Preferred pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Pharmaceutical compositions suitable for injection include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. Isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride may be included in the pharmaceutical composition. In all cases, the composition should be sterile and should be fluid. It should be stable under the conditions of manufacture and storage and must include preservatives that prevent contamination with microorganisms such as bacteria and fungi. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants.

Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like.

Compositions are preferably formulated in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form refers to physically discrete units suited as unitary dosages for a mammalian subject; each unit contains a predetermined quantity of active material (*e.g.*, the nucleic acid vaccine) calculated to produce the desired therapeutic effect, in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of, and sensitivity of, individual subjects

For lung instillation, aerosolized solutions are used. In a sprayable aerosol preparations, the active protein may be in combination with a solid or liquid inert carrier material. This may also be packaged in a squeeze bottle or in admixture with a pressurized volatile, normally

gaseous propellant. The aerosol preparations can contain solvents, buffers, surfactants, and antioxidants in addition to the protein of the invention.

Other pharmaceutically acceptable carriers for the nucleic acid vaccine compositions according to the present invention are liposomes, pharmaceutical compositions in which the active protein is contained either dispersed or variously present in corpuscles consisting of aqueous concentric layers adherent to lipidic layers. The active protein is preferably present in the aqueous layer and in the lipidic layer, inside or outside, or, in any event, in the non-homogeneous system generally known as a liposomic suspension. The hydrophobic layer, or lipidic layer, generally, but not exclusively, comprises phospholipids such as lecithin and sphingomyelin, steroids such as cholesterol, more or less ionic surface active substances such as dicetylphosphate, stearylamine or phosphatidic acid, and/or other materials of a hydrophobic nature. Those skilled in the art will appreciate other suitable embodiments of the present liposomal formulations.

ANTIGENS ASSOCIATED WITH PATHOGENS

A major use for the present invention is the use of the present nucleic acid compositions in therapeutic vaccine for cancer and for major chronic viral infections that cause morbidity and mortality worldwide. Such vaccines are designed to eliminate infected cells - this requires T cell responses as antibodies are often ineffective. The vaccines of the present invention are designed to meet these needs.

Preferred antigens are epitopes of pathogenic microorganisms against which the host is defended by effector T cells responses, including cytotoxic T lymphocyte (CTL) and delayed type hypersensitivity. These typically include viruses, intracellular parasites such as malaria, and bacteria that grow intracellularly such as Mycobacteria and Listeria species. Thus, the types of antigens included in the vaccine compositions of this invention are any of those associated with such pathogens (in addition, of course, to tumor-specific antigens). It is noteworthy that some viral antigens are also tumor antigens in the case where the virus is a causative factor in cancer.

In fact, the two most common cancers worldwide, hepatoma and cervical cancer, are associated with viral infection. Hepatitis B virus(HBV) (Beasley, R.P. *et al.*, *Lancet* **2**, 1129-1133 (1981) has been implicated as etiologic agent of hepatomas. 80-90% of cervical cancers express the E6 and E7 antigens (exemplified herein) from one of four "high risk" human papillomavirus types: HPV-16, HPV-18, HPV-31 and HPV-45 (Gissmann, L. *et al.*, *Ciba Found*

Symp. **120**, 190-207 (1986); Beaudenon, S., *et al. Nature* **321**, 246-249 (1986). The HPV E6 and E7 antigens are the most promising targets for virus associated cancers in immunocompetent individuals because of their ubiquitous expression in cervical cancer. In addition to their importance as targets for therapeutic cancer vaccines, virus associated tumor antigens are also ideal candidates for prophylactic vaccines. Indeed, introduction of prophylactic HBV vaccines in Asia have decrcased the incidence of hepatoma (Chang, M.H., *et al. New Engl. J. Med.* **336**, 1855-1859 (1997), representing a great impact on cancer prevention.

Among the most important viruses in chronic human viral infections are HPV, HBV, hepatitis C Virus (HCV), human immunodeficiency virus (HIV-1 and HIV-2), herpesviruses such as Epstein Barr Virus (EBV),cytomegalovirus (CMV) and HSV-1 and HSV-2 and influenza virus. Useful antigens include HBV surface antigen or HBV core antigen; ppUL83 or pp89 of CMV; antigens of gp120, gp41 or p24 proteins of HIV-1; ICP27, gD2, gB of HSV; or influenza nucleoprotein (Anthony, LS *et al., Vaccine* 1999; 17:373-83). Other antigens associated with pathogens that can be utilized as described herein are antigens of various parasites, includes malaria, preferably malaria peptide (NANP)40.

In addition to its applicability to human cancer and infectious diseases,, the present invention is also intended for use in treating animal diseases in the veterinary medicine context. Thus, the approaches described herein may be readily applied by one skilled in the art to treatment of veterinary herpesvirus infections including equine herpesviruses, bovine viruses such as bovine viral diarrhea virus (for example, the E2 antigen), bovine herpesviruses, Marek's disease virus in chickens and other fowl; animal retroviral and lentiviral diseases (*e.g.*, feline leukemia, feline immunodeficiency, simian immunodeficiency viruses, *etc.*); pseudorabies and rabies; and the like.

As for tumor antigens, any tumor-associated or tumor-specific antigen that can be recognized by T cells, preferably by CTL, can be used. In addition to the HPV-E7 antigen exemplified herein is mutant p53 or HER2/neu or a peptide thereof. Any of a number of melanoma-associated antigens may be used, such as MAGE-1, MAGE-3, MART-1/Melan-A, tyrosinase, gp75, gp100, BAGE, GAGE-1, GAGE-2, GnT-V, and p15 (see, US 6,187,306).

The following references set forth principles and current information in the field of basic, medical and veterinary virology and are incorporated by reference: *Fields Virology*, Fields, BN *et al.*, eds., Lippincott Williams & Wilkins, NY, 1996; *Principles of Virology: Molecular*

Biology, Pathogenesis, and Control, Flint, S.J. *et al.*, eds., Amer Society for Microbiology, Washington, 1999; *Principles and Practice of Clinical Virology*, 4th Edition, Zuckerman. A.J. *et al.*, eds, John Wiley & Sons, NY, 1999; *The Hepatitis C Viruses*, by Hagedorn, CH *et al.*, eds., Springer Verlag, 1999; *Hepatitis B Virus: Molecular Mechanisms in Disease and Novel Strategies for Therapy*, Koshy, R. *et al.*, eds,, World Scientific Pub Co, 1998; *Veterinary Virology*, Murphy, F.A. *et al.*, eds., Academic Press, NY, 1999; *Avian Viruses: Function and Control*, Ritchic, B.W., Iowa State University Press, Ames , 2000; *Virus Taxonomy: Classification and Nomenclature of Viruses: Seventh Report of the International Committee on Taxonomy of Viruses*, by M. H. V. Van Regenmortel, MHV *et al.*, eds., Academic Press; NY, 2000.

DELIVERY OF VACCINE NUCLEIC ACID TO CELLS AND ANIMALS

The Examples below describe certain preferred approaches to delivery of the vaccines of the present invention. A broader description of other approaches including viral and nonviral vectors and delivery mechanisms follow.

DNA delivery involves introduction of a "foreign" DNA into a cell *ex vivo* and ultimately, into a live animal or directly into the animal. Several general strategies for gene delivery (= delivery of nucleic acid vectors) for purposes that include "gene therapy" have been studied and reviewed extensively (Yang, N-S., *Crit. Rev. Biotechnol. 12*:335-356 (1992); Anderson, W.F., *Science 256*:808-813 (1992); Miller, A.S., *Nature 357*:455-460 (1992); Crystal, R.G., *Amer. J. Med. 92(suppl 6A)*:44S-52S (1992); Zwiebel, J.A. *et al.*, *Ann. N.Y. Acad. Sci. 618*:394-404 (1991); McLachlin, J.R. *et al.*, *Prog. Nucl. Acid Res. Molec. Biol. 38*:91-135 (1990); Kohn, D.B. *et al.*, *Cancer Invest. 7*:179-192 (1989), which references are herein incorporated by reference in their entirety).

One approach comprises nucleic acid transfer into primary cells in culture followed by autologous transplantation of the *ex vivo* transformed cells into the host, either systemically or into a particular organ or tissue.

The term "systemic administration" refers to administration of a composition or agent such as a molecular vaccine as described herein, in a manner that results in the introduction of the composition into the subject's circulatory system or otherwise permits its spread throughout the body. "Regional" administration refers to administration into a specific, and somewhat more limited, anatomical space, such as intraperitoneal, intrathecal, subdural, or to a specific organ.

The term "local administration" refers to administration of a composition or drug into a limited, or circumscribed, anatomic space, such as intratumoral injection into a tumor mass, subcutaneous injections, intramuscular injections. One of skill in the art would understand that local administration or regional administration may also result in entry of a composition into the circulatory system.

For accomplishing the objectives of the present invention, nucleic acid therapy would be accomplished by direct transfer of a the functionally active DNA into mammalian somatic tissue or organ *in vivo*. DNA transfer can be achieved using a number of approaches described below. These systems can be tested for successful expression *in vitro* by use of a selectable marker (*e.g.*, G418 resistance) to select transfected clones expressing the DNA, followed by detection of the presence of the antigen-containing expression product (after treatment with the inducer in the case of an inducible system) using an antibody to the product in an appropriate immunoassay. Efficiency of the procedure, including DNA uptake, plasmid integration and stability of integrated plasmids, can be improved by linearizing the plasmid DNA using known methods, and co-transfection using high molecular weight mammalian DNA as a "carrier".

Examples of successful "gene transfer" reported in the art include: (a) direct injection of plasmid DNA into mouse muscle tissues, which led to expression of marker genes for an indefinite period of time (Wolff, J.A. *et al.*, *Science 247*:1465 (1990); Acsadi, G. *et al.*, *The New Biologist 3*:71 (1991)); (b) retroviral vectors are effective for *in vivo* and *in situ* infection of blood vessel tissues; (c) portal vein injection and direct injection of retrovirus preparations into liver effected gene transfer and expression *in vivo* (Horzaglou, M. *et al.*, *J. Biol. Chem. 265*:17285 (1990); Koleko, M. *et al.*, *Human Gene Therapy 2*:27 (1991); Ferry, N. *et al.*, *Proc. Natl. Acad. Sci. USA* 88:8387 (1991)); (d) intratracheal infusion of recombinant adenovirus into lung tissues was effective for *in vivo* transfer and prolonged expression of foreign genes in lung respiratory epithelium (Rosenfeld, M.A. *et al.*, *Science 252*:431 (1991); (e) Herpes simplex virus vectors achieved *in vivo* gene transfer into brain tissue (Ahmad, F. *et al.*, eds, *Miami Short Reports - Advances in Gene Technology: The Molecular Biology of Human Genetic Disease*, Vol 1, Boehringer Manneheim1 Biochemicals, USA, 1991).

Retroviral-mediated human therapy utilizes amphotrophic, replication-deficient retrovirus systems (Temin, H.M., *Human Gene Therapy 1*:111 (1990); Temin *et al.*, U.S. Patent 4,980,289; Temin *et al.*, U.S. Patent 4,650,764; Temin *et al.*, U.S. Patent No. 5,124,263; Wills,

J.W. U.S. Patent 5,175,099; Miller, A.D., U.S. Patent No. 4,861,719). Such vectors have been used to introduce functional DNA into human cells or tissues, for example, the adenosine deaminase gene into lymphocytes, the NPT-II gene and the gene for tumor necrosis factor into tumor infiltrating lymphocytes. Retrovirus-mediated gene delivery generally requires target cell proliferation for gene transfer (Miller, D.G. *et al.*, *Mol. Cell. Biol. 10*:4239 (1990). This condition is met by certain of the preferred target cells into which the present DNA molecules are to be introduced, *i.e.*, actively growing tumor cells. Gene therapy of cystic fibrosis using transfection by plasmids using any of a number of methods and by retroviral vectors has been described by Collins *et al.*, U.S. Patent 5,240,846.

The DNA molecules encoding the fusion polypeptides of the present invention may be packaged into retrovirus vectors using packaging cell lines that produce replication-defective retroviruses, as is well-known in the art (see, for example, Cone, R.D. *et al.*, *Proc. Natl. Acad. Sci. USA* 81:6349-6353 (1984); Mann, R.F. *et al.*, *Cell* 33:153-159 (1983); Miller, A.D. *et al.*, *Molec. Cell. Biol.* 5:431-437 (1985),; Sorge, J., *et al.*, *Molec. Cell. Biol.* 4:1730-1737 (1984); Hock, R.A. *et al.*, *Nature* 320:257 (1986); Miller, A.D. *et al.*, *Molec. Cell. Biol.* 6:2895-2902 (1986). Newer packaging cell lines which are efficient an safe for gene transfer have also been described (Bank *et al.*, U.S. 5,278,056.

This approach can be utilized in a site specific manner to deliver the retroviral vector to the tissue or organ of choice. Thus, for example, a catheter delivery system can be used (Nabel, EG *et al.*, *Science 244*:1342 (1989)). Such methods, using either a retroviral vector or a liposome vector, are particularly useful to deliver the nucleic acid to be expressed to a blood vessel wall, or into the blood circulation of a tumor.

Other virus vectors may also be used, including recombinant adenoviruses (Horowitz, M.S., In: *Virology*, Fields, BN *et al.*, eds, Raven Press, New York, 1990, p. 1679; Berkner, K.L., *Biotechniques* 6:616 9191988), Strauss, S.E., In: *The Adenoviruses*, Ginsberg, HS, ed., Plenum Press, New York, 1984, chapter 11), herpes simplex virus (HSV) for neuron-specific delivery and persistence. Advantages of adenovirus vectors for human gene delivery include the fact that recombination is rare, no human malignancies are known to be associated with such viruses, the adenovirus genome is double stranded DNA which can be manipulated to accept foreign genes of up to 7.5 kb in size, and live adenovirus is a safe human vaccine organisms. Adeno-

associated virus is also useful for human therapy (Samulski, R.J. *et al.*, *EMBO J. 10*:3941 (1991) according to the present invention.

Another vector which can express the DNA molecule of the present invention, and is useful in the present therapeutic setting, particularly in humans, is vaccinia virus, which can be rendered non-replicating (U.S. Patents 5,225,336; 5,204,243; 5,155,020; 4,769,330; Sutter, G *et al.*, *Proc. Natl. Acad. Sci. USA* (1992) *89*:10847-10851; Fuerst, T.R. *et al.*, *Proc. Natl. Acad. Sci. USA* (1989) *86*:2549-2553; Falkner F.G. *et al.*; *Nucl. Acids Res* (1987) *15*:7192; Chakrabarti, S *et al.*, *Molec. Cell. Biol.* (1985) *5*:3403-3409). Descriptions of recombinant vaccinia viruses and other viruses containing heterologous DNA and their uses in immunization and DNA therapy are reviewed in: Moss, B., *Curr. Opin. Genet. Dev.* (1993) *3*:86-90; Moss, B. *Biotechnology* (1992) *20*:345-362; Moss, B., *Curr Top Microbiol Immunol* (1992) *158*:25-38; Moss, B., *Science* (1991) *252*:1662-1667; Piccini, A *et al.*, *Adv. Virus Res.* (1988) 34:43-64; Moss, B. *et al.*, *Gene Amplif Anal* (1983) 3:201-213.

In addition to naked DNA or RNA, or viral vectors, engineered bacteria may be used as vectors. A number of bacterial strains including *Salmonella*, BCG and *Listeria monocytogenes*(LM) (Hoiseth & Stocker, *Nature* **291**, 238-239 (1981); Poirier, TP *et al. J. Exp. Med.* **168**, 25-32 (1988); (Sadoff, J.C., *et al., Science* **240**, 336-338 (1988); Stover, C.K., *et al., Nature* **351**, 456-460 (1991); Aldovini, A. *et al.,, Nature* **351**, 479-482 (1991); Schafer, R., *et al., J. Immunol.* **149**, 53-59 (1992); Ikonomidis, G. *et al., J. Exp. Med.* **180**, 2209-2218 (1994)). These organisms display two promising characteristics for use as vaccine vectors: (1) enteric routes of infection, providing the possibility of oral vaccine delivery; and (2) infection of monocytes/macrophages thereby targeting antigens to professional APCs.

In addition to virus-mediated gene transfer *in vivo*, physical means well-known in the art can be used for direct transfer of DNA, including administration of plasmid DNA (Wolff *et al.*, 1990, *supra*) and particle-bombardment mediated gene transfer (Yang, N.-S., *et al.*, *Proc. Natl. Acad. Sci. USA 87*:9568 (1990); Williams, R.S. *et al.*, *Proc. Natl. Acad. Sci. USA 88*:2726 (1991); Zelenin, A.V. *et al.*, *FEBS Lett. 280*:94 (1991); Zelenin, A.V. *et al.*, *FEBS Lett. 244*:65 (1989); Johnston, S.A. *et al.*, *In Vitro Cell. Dev. Biol. 27*:11 (1991)). Furthermore, electroporation, a well-known means to transfer genes into cell *in vitro*, can be used to transfer DNA molecules according to the present invention to tissues *in vivo* (Titomirov, A.V. *et al.*, *Biochim. Biophys. Acta 1088*:131 ((1991)).

"Carrier mediated gene transfer" has also been described (Wu, C.H. *et al.*, *J. Biol. Chem.* *264*:16985 (1989); Wu, G.Y. *et al.*, *J. Biol. Chem. 263*:14621 (1988); Soriano, P. *et al.*, *Proc. Natl. Acad. Sci. USA 80*:7128 (1983); Wang, C-Y. *et al.*, *Proc. Natl. Acad. Sci. USA 84*:7851 (1982); Wilson, J.M. *et al.*, *J. Biol. Chem. 267*:963 (1992)). Preferred carriers are targeted liposomes (Nicolau, C. *et al.*, *Proc. Natl. Acad. Sci. USA 80*:1068 (1983); Soriano *et al.*, *supra*) such as immunoliposomes, which can incorporate acylated mAbs into the lipid bilayer (Wang *et al.*, *supra*). Polycations such as asialoglycoprotein/polylysine (Wu *et al.*, 1989, *supra*) may be used, where the conjugate includes a molecule which recognizes the target tissue (e.g., asialoorosomucoid for liver) and a DNA binding compound to bind to the DNA to be transfected. Polylysine is an example of a DNA binding molecule which binds DNA without damaging it. This conjugate is then complexed with plasmid DNA according to the present invention for transfer.

Plasmid DNA used for transfection or microinjection may be prepared using methods well-known in the art, for example using the Quiagen procedure (Quiagen), followed by DNA purification using known methods, such as the methods exemplified herein.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

EXAMPLE I

Materials and Methods

(These examples incorporate by reference C-F Hung et al., Canc Res. 61:3698-3703 (2001))

Plasmid DNA Constructs and Preparation

The generation of pcDNA3-E7 has been described previously (5). For the generation of pcDNA3-ETA(dII), the pGW601 plasmid (Wozniak, DJ *et al.*, Proc Natl Acad Sci U S A. *85:* 8880-8884, 1988) (provided by Dr. Darrell R. Galloway at Ohio State University) was used as the template for amplification of ETA(dII). The DNA fragment containing ETA(dII) was generated using PCR with a set of primers: 5'-ccgggaattcatgcgcctgcactttcccgagggc-3' (SEQ ID NO:10) and 5'-ccggaattcgttctgcgtgccgcgggtgctgaa-3'. (SEQ ID NO:11)

The amplified DNA fragment was then cloned into the EcoRI site of pcDNA3 (Invitrogen, Carlsbad, CA). For the generation of pcDNA3-ETA(dII)/E7, the DNA fragment containing

ETA(dII) DNA was cloned into the EcoRI site of pcDNA3-E7. For the generation of pcDNA3-GFP, DNA fragment encoding the green fluorescent protein (GFP) was first amplified with PCR using pEGFPN1 DNA (Clontech, Palo Alto, CA) and a set of primers:

5'-atcggatccatggtgagcaagggcgaggag-3' (SEQ ID NO:12) and

5'-gggaagctttacttgtacagctcgtccatg-3'. (SEQ ID NO:13).

The amplified product was then cloned into the BamHI/HindIII cloning sites of pcDNA3. For the generation of pcDNA3-E7/GFP, E7 was subcloned from pcDNA3-E7 into the EcoRI/BamHI sites of pcDNA3-GFP. For the generation of pcDNA3-ETA(dII)/E7/GFP, the ETA(dII) fragment was amplified using

5'-gggtctagaatgcgcctgcactttcccgagggc-3' (SEQ ID NO:14) and

5'-ccggaattcgttctgcgtgccgcgggtgctgaa-3' (SEQ ID NO:15)

as primers and cloned into the XbaI/EcoRI sites of pcDNA3-E7/GFP. The accuracy of all the constructs was confirmed by DNA sequencing. DNA for vaccination was prepared using an endotoxin-free kit (Qiagen, Valencia, CA).

Western Blot Analysis

20 μg of pcDNA3, E7, or ETA(dII)/E7 DNA were transfected into $5x10^6$ 293 D^bK^b cells (Bloom, MB *et al.*, J Exp Med. *185:* 453-459, 1997) using lipofectamine 2000 (Life Technologies, Rockville, MD). 24 hr after transfection, cell lysates were used for Western blot analysis. Equal amounts of proteins (50 μg) were separated by SDS-PAGE using a 12% polyacrylamide gel and were electroblotted to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). Membranes were probed with E7-specific mouse monoclonal antibody (Zymed, San Francisco, CA) at a concentration of 0.25 μg/ml and then incubated with goat anti-mouse IgG conjugated to alkaline phosphatase (1:1000 dilution; Amersham, Piscataway, NJ). Membranes were treated with ECL and developed using Hyperfilm-ECL (Amersham, Piscataway, NJ).

Mice: 6- to 8-week old female C57BL/6 mice from the National Cancer Institute (Frederick, Maryland) were purchased and kept in the oncology animal facility of the Johns Hopkins Hospital (Baltimore, Maryland). All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

CTL Assay using Transfected 293 D^bK^b Cells as Target Cells

A human embryonic kidney 293 cell line expressing the D^b and K^b (293 D^bK^b) two C57BL/6 mouse MHC class I molecules, was kindly provided by Dr. JC Yang (National Cancer Institute, National Institutes of Health, Bethesda). 20μg of pcDNA3 (no insert), ETA(dII), E7, or ETA(dII)/E7 DNA were transfected into $5x10^6$ 293 D^bK^b cells using lipofectamine 2000 (Life Technologies, Rockville, MD). Cells were collected 40-44 hr after transfection. Transfected 293 D^bK^b cells (Bloom *et al.*, *supra*) were used as target cells while a D^b-restricted E7-specific $CD8^+$ T cell line (Wang, T-L *et al.*, Gene Therapy. *7:* 726-733, 2000) served as effector cells. Untransfected 293 D^bK^b cells were used as a negative control. Cytolysis was determined by quantitative measurements of lactate dehydrogenase (LDH) using CytoTox96, non-radioactive cytotoxicity assay kits (Promega, Madison, WI) according to the manufacturer's protocol. CTL assays were performed with effector cells and targets cells (10^4 per well) mixed together at various ratios (1:1, 3:1, 9:1, and 27:1) in a final volume of 200 μl. After a 5 hr incubation at 37°C, 50 μl of the cultured media were collected to assess the amount of LDH in the cultured media. The percentage of lysis was calculated from the following equation:

% Lysis = 100 x (A - B)/(C - D)

where A is the reading of experimental-effector signal value, B is the effector spontaneous background signal value, C is maximum signal value from target cells, D is the target spontaneous background signal value.

CTL Assay Using DCs Pulsed with Lysates of Transfected 293 D^bK^b Cells as Target Cells

CTL assays were performed with freshly isolated bone marrow-derived DCs pulsed with cell lysates as target cells and E7-specific $CD8^+$ T cells as effector cells using a protocol similar to that described previously (Lu, Z *et al.*, J Exp Med. *191:* 541-550, 2000). The protein concentration was determined using the BioRad protein assay (Bio-Rad, Hercules, CA) according to vendor's protocol. 293 D^bK^b cells were transfected as described earlier. Cell lysates from E7 or ETA(dII)/E7 DNA-transfected 293 D^bK^b cells were standardized for E7 protein concentration using an ELISA. DCs were prepared by pulsing them with different concentrations of cell lysates of various DNA-transfected 293 D^bK^b cells (50 μg/ml, 10 μg/ml, 2 μg/ml and 0.4 μg/ml) in a final volume of 2 ml for 16-20 hrs. CTL assays were performed at a fixed E/T (9/1) ratio with $9x10^4$ E7-specific T cells mixed with $1x10^4$ prepared DCs in a final

volume of 200 µl. Cytolysis was determined by quantitative measurements of LDH as described earlier.

DNA Vaccination

Preparation of DNA-coated gold particles and gene gun particle-mediated DNA vaccination was performed using a helium-driven gene gun (Bio-Rad, Hercules, CA) according to a previously described protocol (5). DNA-coated gold particles (1 µg DNA/bullet) were delivered to the shaved abdominal region of mice using a helium-driven gene gun (Bio-Rad, Hercules, CA) with a discharge pressure of 400 p.s.i.

Intracytoplasmic Cytokine Staining and Flow Cytometry Analysis

Cell surface marker staining of CD8 or CD4 and intracellular cytokine staining for IFN-γ and IL-4 as well as FACScan analysis was performed using conditions described previously (Ji *et al.*, 1999, *supra*). Prior to FACScan, splenocytes from naïve or vaccinated groups of mice were incubated for 20 hours with either 1 µg/ml of E7 peptide (aa 49-57) containing MHC class I epitope for detecting E7-specific $CD8^+$ T cell precursors or 10 µg/ml of E7 peptide (aa 30-67) containing MHC class II peptide for detecting E7-specific $CD4^+$ T cell precursors.

ELISA

For detection of HPV-16 E7-specific antibodies in the sera of vaccinated mice, we performed a direct ELISA with 1:100, 1:500, and 1: 1000 dilutions of sera in 1x PBS as previously described (Wu, T-C *et al.*, Proc. Natl. Acad. Sci. *92:* 11671-11675, 1995). Briefly, sera was added to microwell plates coated with bacteria-derived HPV-16 E7 proteins followed by incubation with peroxidase-conjugated rabbit anti-mouse IgG antibody (Zymed, San Francisco, CA).

In Vivo Tumor Protection Experiments

For the tumor protection experiment, mice (5 per group) were vaccinated via gene gun with 2 µg of pcDNA3 without insert, ETA(dII) DNA, E7 DNA, ETA(dII) mixed with E7, or chimeric ETA(dII)/E7 DNA. One week later, the mice were boosted with the same regimen as the first vaccination. One week after the last vaccination, mice were subcutaneously challenged with 5 x 10^4 cells/mouse TC-1 tumor cells (Lin *et al.*, *supra*) in the right leg and then monitored twice a week.

In Vivo Tumor Treatment Experiments

Mice were intravenously challenged with 10^4 cells/mouse TC-1 tumor cells via tail vein on day 0. Three days after challenge with TC-1 tumor cells, mice treated with 2 μg of pcDNA3 without insert, ETA(dII) DNA, E7 DNA, or chimeric ETA(dII)/E7 DNA via gene gun. One week later, these mice were boosted with the same regimen as the first vaccination. Mice were sacrificed on day 25. The number of pulmonary metastatic nodules of each mouse was evaluated and counted by experimenters blinded to sample identity.

In Vivo Antibody Depletion Experiments

In vivo antibody depletions were performed as described previously (Lin *et al.*, *supra.*) Briefly, mice were vaccinated with 2 μg ETA(dII)/E7 DNA via gene gun, boosted one week later, and challenged with 5 x 10^4 cells/mouse TC-1 tumor cells subcutaneously. Depletions were started one week prior to tumor challenge. MAb GK1.5 was used for CD4 depletion, MAb 2.43 was used for CD8 depletion, and MAb PK136 was used for NK1.1 depletion. Depletion was terminated on day 63 after tumor challenge.

EXAMPLE II

Generation and Characterization of the ETA(dII)/E7 DNA Vaccine

A schematic diagram showing the domains of full-length ETA and the construct of chimeric ETA(dII)/E7 is presented in **Fig. 1A**. Chimeric ETA(dII)/E7 was created by linking ETA(dII) (aa 247-416) to the E7 protein. We performed a Western blot analysis to characterize protein expression in E7 and ETA(dII)/E7 DNA-transfected cells (**Fig. 1B**). Analysis of lysates of E7 DNA-transfected 293 D^bK^b cells revealed a protein band with a size of approximately 15 kDa corresponding to E7 protein. Analysis of lysates of ETA(dII)/E7 DNA-transfected 293 D^bK^b cells generated a protein band with a size of approximately 35 kDa corresponding to ETA(dII)/E7 protein. Immunoblotting with an E7-specific antibody indicated that ETA(dII)/E7 DNA-transfected cells exhibited similar levels of E7 protein expression compared to E7 DNA-transfected cells (**Fig. 1B**).

EXAMPLE III

Enhanced Presentation of E7 Through MHC Class I Pathway in Cells Transfected with ETA(dII)/E7 DNA

To test whether addition of the translocation domain of ETA to E7 can directly enhance MHC class I presentation of E7, we performed CTL assays to characterize the MHC class I

presentation of E7 by 293 D^bK^b cells transfected with various DNA constructs. We chose 293 D^bK^b cells as target cells because they have a stable high transfection efficiency (up to 80%) and high expression of the C57BL/6 MHC class I D^b molecule. T cells of A D^b-restricted E7-specific $CD8^+$ T cell line (26) served as effector cells. As shown in **Fig. 2A**, 293 D^bK^b cells transfected with ETA(dII)/E7 DNA were killed at a significantly higher level at the 9:1 E:T ratio (33.3±3.3% versus 12.5±1.1%, $p<0.001$) and 27:1 (62.1±6.0% versus 22.6±3.0%, $p<0.001$) compared to cells transfected with wild-type E7 DNA. These results indicate that cells transfected with ETA(dII)/E7 DNA present E7 antigen through the MHC class I pathway more efficiently than cells transfected with wild-type E7 DNA.

EXAMPLE IV

Enhanced Presentation of E7 Through the MHC Class I Pathway in Dendritic Cells Pulsed With Lysates of Cells Transfected With Chimeric ETA(dII)/E7 DNA

To demonstrate if the addition of the translocation domain of ETA to E7 can lead to enhanced MHC class I presentation of E7 via a "cross-priming" mechanism (Huang, AY *et al.*, Science. *264:* 961-965, 1994), we performed CTL assays to characterize the MHC class I presentation of E7 using bone marrow-derived DCs pulsed with cell lysates of 293 D^bK^b cells transfected with various DNA constructs. As shown in **Fig. 2B**, DCs pulsed with lysates of 293 D^bK^b cells transfected with ETA(dII)/E7 DNA were lysed at significantly higher levels compared to (1) DCs pulsed with lysates of 293 D^bK^b cells transfected with the other DNA constructs or (2) naïve DCs ($p<0.001$). These results revealed that the fusion of ETA(dII) to E7 enhances MHC class I presentation of E7 via a "cross-priming" mechanism.

EXAMPLE V

Significant Enhancement of E7-Specific $CD8^+$ T Cell Precursors in Mice Vaccinated with ETA(dII)/E7 DNA

To determine whether mice vaccinated with various DNA vaccine constructs generate E7-specific $CD8^+$ T cell precursors, we performed intracellular cytokine staining to detect E7-specific $CD8^+$ T cell precursors in spleens of vaccinated mice (5). As shown in **Fig. 3A**, mice vaccinated with ETA(dII)/E7 DNA generated an approximately 30-fold increase in the number of E7-specific IFN-γ^+ $CD8^+$ T cell precursors (308/3 x 10^5 splenocytes) compared to mice vaccinated with E7 DNA (11/3 x 10^5 splenocytes) ($p<0.01$). Fusion of ETA(dII) to E7 was

required for enhancement of E7-specific $CD8^+$ T cell activity because a mixture of ETA(dII) with E7 ("ETA(dII)+E7 DNA" group) did not generate enhanced $CD8^+$ T cell activity. Furthermore, the linkage of irrelevant proteins (such as GFP and CTLA-4) to E7 did not generate enhanced E7-specific CD8+ T cell activity.

No significant differences were observed in the number of E7-specific $CD4^+$ IFN-γ^+ T cells (**Fig. 3B**) or $CD4^+$ IL-4^+ T cells among each of the vaccination groups. No significant enhancement of E7-specific antibody responses was observed in mice vaccinated with ETA(dII)/E7 DNA compared to the other vaccination groups using ELISA.

EXAMPLE VI

Vaccination with ETA(dII)/E7 Fusion DNA Enhances Protection Against the Growth of E7-Expressing Tumors

To determine if the observed enhancement in E7-specific $CD8^+$ T cell-mediated immunity translated to a significant E7-specific antitumor effect, we performed *in vivo* tumor protection studies using a previously characterized E7-expressing tumor model, TC-1 (4). As shown in Fig. 4A, 100% of mice vaccinated with ETA(dII)/E7 DNA remained tumor-free at 56 days after TC-1 challenge, while all other groups developed tumors within 15 days of challenge. Here again, fusion of ETA(dII) to E7 was required for antitumor immunity, because ETA(dII) mixed with E7 ("ETA(dII)+E7 DNA") did not enhance antitumor immunity. Furthermore, the linkage of irrelevant proteins (such as GFP and CTLA-4) to E7 did not enhance tumor protection.

EXAMPLE VII

Treatment with ETA(dII)/E7 Fusion DNA Eradicates Established E7-Expressing Tumors in the Lungs

To determine the therapeutic potential of chimeric ETA(dII)/E7 DNA in treating TC-1 tumor metastases in the lungs, an *in vivo* tumor treatment experiment was performed using a lung metastasis model (Ji, H *et al.*, Int J Cancer. *78:* 41-45, 1998). As shown in **Fig. 4B**, mice vaccinated with ETA(dII)/E7 DNA revealed the lowest mean number of pulmonary nodules (1.6±1.1) compared to mice vaccinated with wild-type E7 DNA (77.6±22.1), or ETA(dII) DNA (73.4±14.6) (one-way ANOVA, $p<0.001$). These results show that treatment with ETA(dII)/E7 could control and eradicate established E7-expressing tumors in the lungs.

EXAMPLE VIII

$CD8^+$ T Cells But Not $CD4^+$ T cells or NK cells are Essential for the Antitumor Effect of Chimeric ETA(dII)/E7 DNA

To determine the class of classes of lymphocytes participating in the rejection of E7+ tumor cells stimulated by the vaccine, we performed *in vivo* antibody depletion experiments. As shown in **Fig. 4C**, all naïve (unvaccinated) mice and all vaccinated mice depleted of $CD8^+$ T cells grew tumors within 14 days after challenge. In contrast, all non-depleted mice and all mice depleted of $CD4^+$ T cells or NK1.1+ cells remained tumor-free 60 days after tumor challenge. These results suggest that $CD8^+$ T cells, but not $CD4^+$ T cells or NK cells, are essential for the anti-tumor immunity induced by the ETA(dII)/E7 DNA vaccine.

DISCUSSION OF EXAMPLES II-VIII

Direct linkage of ETA(dII) to E7 dramatically enhanced the potency of HPV-16 E7-containing DNA vaccines. A DNA vaccine encoding ETA(dII) fused to HPV-16 E7 elicited strong E7-specific $CD8^+$ T cell-mediated immunity and generated significant $CD8^+$ T cell-dependent preventive effects against HPV-16 E7-expressing murine tumors. Furthermore, the chimeric ETA(dII)/E7 DNA vaccine was capable of inducing a state of immunity that successfully controlled lethal metastatic lung tumors.

The vaccine of this invention represents a successful case of employing the translocation domain of a bacterial toxin in the broader context of "gene therapy." Others employed ETA(dII) linked to a DNA-binding protein to facilitate the entry of DNA into the cytosol (Fominaya, J *et al.*, 1996 and 1998, *supra*). Truncated forms of this chimeric protein lacking the translocation domain failed to facilitate efficient DNA transfer. These studies suggested the utility of ETA(dII) for introducing exogenous DNA (to be expressed as protein) into the cytosol. The precise mechanism of such translocation nevertheless remains unclear.

One explanation for the observed enhancement of E7-specific $CD8^+$ T cell activity in mice vaccinated with ETA(dII)/E7 DNA is enhanced MHC class I presentation of E7 in cells expressing this chimeric protein. Indeed, in the *in vitro* studies above showed that cells transfected with ETA(dII)/E7 DNA presented E7 through the MHC class I pathway more efficiently than did cells transfected with wild-type E7 DNA (**Fig. 2A**). Since biolistic DNA delivery can introduce DNA directly into professional APCs in the dermis, ETA(dII)/E7 DNA-transfected APCs may directly enhance the presentation of E7 through the MHC class I pathway

to $CD8^+$ T cells and thereby contribute to the proliferation and differentiation of E7-specific $CD8^+$ CTL precursors *in vivo*.

Another important mechanism contributing to the enhanced $CD8^+$ T cell responses *in vivo* is the "cross-priming" effect of the chimeric protein, whereby release of ETA(dII)E7 antigen leads to uptake and re-processing by other APCs via the MHC class I-restricted pathway (Huang *et al.*, *supra*). The present results show that linkage of ETA(dII) to E7 leads to enhanced priming of E7-specific $CD8^+$ T cells presumably via "cross-priming" (**Fig. 2B**). One previous report disclosed that exogenous ETA (domains I and II) fused to influenza A protein or nucleoprotein resulted in MHC class I processing and presentation of the antigen to CTLs (Donnelly, JJ *et al.*, Proc Natl Acad Sci U S A. *90:* 3530-3534, 1993). The present results suggest that linkage to domain II alone is sufficient for delivery of exogenous antigen into the MHC class I presentation pathway.

The success of the ETA(dII)/E7 DNA vaccine described herein, and the importance of domain II in this construct points to strategies using translocation domains of other bacterial toxins to enhance vaccine potency. Translocation domains for several bacterial toxins have are known, including diphtheria toxin (Umata, T *et al.,* J Biol Chem. *273:* 8351-8359, 1998; Oh, KJ *et al.*, Proc Natl Acad Sci U S A. *96:* 8467-8470, 1999), clostridial neurotoxins such as tetanus neurotoxins and botulinum neurotoxins (Finkelstein, A. J Physiol. *84:* 188-190, 1990; Pellizzari, R *et al.*, Philos Trans Roy Soc Lond B Biol Sci. *354:* 259-268, 1999), anthrax toxin lethal factor (Arora, N *et al.*, Infect Immun. *62:* 4955-4961, 1994; Collier, RJ. J Appl Microbiol. *87:* 283-288, 1999), Shiga toxin (Sandvig, K *et al.*, Nature. *358:* 510-512, 1992), *E. coli* heat-labile toxin (Sixma, TK *et al.*, Nature. *355:* 561-564, 1992), Yersinia cytotoxins (YopE and YopH) (Sory, MP *et al.*, Proc Natl Acad Sci U S A. *92:* 11998-12002, 1995), Listeria toxin (listeriolysin O) (Parrisius, J *et al.*, Infect Immun. *51:* 314-319, 1986), and pertussis adenylate cyclase toxin (Karimova, G *et al.*, Proc Natl Acad Sci U S A. *95:* 12532-12537, 1998). Better understanding of these translocation domains may allow such molecules to be incorporated in vaccine designs similar to that described here.

ETA(dII)/E7 stimulated potent E7-specific CD8+ T cell responses through enhanced MHC class I presentation, and the antitumor effect was completely CD4-independent. Interestingly, these features resemble those recently described by the present inventors' group

using a chimeric DNA vaccine that included *Mycobacterium tuberculosis* heat shock protein 70 (HSP70) linked to E7 (Chen, C-H *et al.*, 2000, *supra*).

While the ETA(dII)/E7 targets antigen to the MHC class I presentation pathway for the enhancement of $CD8^+$ T cell activity, other constructs that target antigen to MHC class II presentation pathways may provide enhanced $CD4^+$ T cell responses. This realization raises the notion of co-administration of vaccines that directly enhance MHC class I and class II restricted pathways. The present inventors and their collaborators previously developed a chimeric Sig/E7/LAMP-1 DNA vaccine that uses the LAMP-1 endosomal/lysosomal targeting signal for enhancing the MHC class II presentation pathway of E7 (Ji, H *et al.*, Human Gene Therapy. *10:* 2727-2740, 1999). The ETA(dII)/E7 vaccine of the present invention used in conjunction with a MHC class II-targeting vaccine such as Sig/E7/LAMP-1 may activate multiple arms of the immune system in a synergistic fashion, leading to significantly enhanced $CD4^+$ and $CD8^+$ T cell responses and potent antitumor effects.

In summary, the results disclosed herein provide methods to enhance vaccine potency by linking ETA(dII) to antigen, allowing enhanced stimulation of antigen-specific $CD8^+$ T cells leading to potent antitumor effects *in vivo*. Since a majority of cervical cancers express HPV E7, the present vaccine is useful for the prevention and treatment of HPV-associated tumors. This approach is useful for the control of cancer, infectious diseases and any other conditions where enhanced T cell reactivity, primarily $CD8^+$ T cell reactivity, is associated with prophylactic or therapeutic outcomes.

The references cited above are all incorporated by reference herein, whether specifically incorporated or not.

Citation of the documents herein is not intended as an admission that any of them is pertinent prior art. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicant and does not constitute any admission as to the correctness of the dates or contents of these documents.

WHAT IS CLAIMED IS:

1. A nucleic acid encoding a chimeric or fusion polypeptide which polypeptide comprises:

(a) a first domain comprising a translocation polypeptide; and

(b) a second domain comprising an antigen which comprises at least one antigenic peptide.

2. The nucleic acid of claim 2 wherein the translocation polypeptide is a bacterial toxin translocation polypeptide.

3. The nucleic acid of claim 2 wherein the bacterial toxin translocation polypeptide is domain II of *Pseudomonas aeruginosa* exotoxin A (ETA(dII)).

4. The nucleic acid of claim 1 wherein the encoded translocation polypeptide is SEQ ID NO:3 or a homologue thereof.

5. The nucleic acid of claim 1 that comprises a nucleotide sequence that encodes a translocation polypeptide which sequence is included in SEQ ID NO:1.

6. The nucleic acid of claim 1, wherein the antigenic peptide comprises an epitope that binds to, and is presented on cell surfaces by, MHC class I proteins.

7 The nucleic acid of claim 6 wherein the epitope of the antigenic peptide is between about 8 and about 11 amino acid residues in length.

8. The nucleic acid of claim 1, wherein the antigen

(i) is derived from a pathogen selected from a mammalian cell, a microorganism or a virus; or

(ii) cross-reacts with an antigen of said pathogen.

9. The nucleic acid of claim 8, wherein the virus is a human papilloma virus.

10. The nucleic acid of claim 9, wherein the antigen is an HPV-16 E7 or E6 peptide.

11. The nucleic acid of claim 8, wherein the antigen is a non-oncogenic HPV-16 E7 polypeptide.

12. The nucleic acid of claim 8, wherein the pathogen is a bacterium.

13. The nucleic acid of claim 1, wherein the antigen is a tumor-specific or tumor-associated antigen.

14. The nucleic acid of claim 1 operatively linked to a promoter.

15. The nucleic acid of claim 14, wherein the promoter is one which is expressed in an antigen presenting cell (APC).

16. The nucleic acid of claim 15, wherein the APC is a dendritic cell.

17. An expression vector comprising

(a) the nucleic acid of any of claims 1-16 operatively linked to

(b) a promoter and, optionally, to one or more regulatory elements that enhance expression of said nucleic acid in a cell.

18. The expression vector of claim 17 which is a viral vector or a plasmid.

19. The expression vector of claim 17 which is a self-replicating RNA replicon.

20. The expression vector of claim 17 wherein the translocation polypeptide is ETA(dII).

21. A particle comprising the nucleic acid of any of claims 1-16.

22. A particle comprising the expression vector of claim 17.

23. The particle of claim 21 which comprises a material is suitable for introduction into a cell or an animal by particle bombardment.

24. The particle of claim 23, wherein the material is gold.

25. A cell which has been modified to comprise the nucleic acid of any of claims 1-16.

26. A cell which has been modified to comprise the expression vector of claim 17, and which cell expresses said nucleic acid.

27. The cell of claim 26 which is an APC.

28. The cell of claim 27, wherein the APC is a dendritic cell, a keratinocyte, a macrophage, a monocyte, a B lymphocyte, a microglial cell, an astrocyte, or an activated endothelial cell.

29. A chimeric polypeptide comprising

(a) a first domain comprising a translocation polypeptide; and

(b) a second domain comprising an antigen which comprises at least one antigenic peptide.

30. The chimeric polypeptide of claim 29 wherein the translocation polypeptide is a bacterial toxin translocation polypeptide.

31. The chimeric polypeptide of claim 30 wherein the bacterial toxin translocation polypeptide is ETA(dII).

32. The chimeric polypeptide of claim 29 wherein the translocation polypeptide is SEQ ID NO:3 or a homologue thereof.

33. A chimeric polypeptide encoded by the nucleic acid of any of claims 1-16.

34. The chimeric polypeptide of any of claims claim 29-32, wherein the antigenic peptide comprises an epitope that binds to, and is presented on cell surfaces by, MHC class I proteins.

35. The chimeric polypeptide of claim 33, wherein the antigenic peptide comprises an epitope that binds to, and is presented on cell surfaces by, MHC class I proteins.

36. The chimeric polypeptide of any of claims 29-32 wherein the translocation polypeptide domain and the antigen are linked by a chemical linker.

37. The chimeric polypeptide of any of claims 29-32 which is a fusion polypeptide.

38. The chimeric polypeptide of claim 33 which is a fusion polypeptide.

39. The chimeric polypeptide of any of claims 29-32 wherein the first domain is N-terminal to the second domain.

40. The chimeric polypeptide of any of claims 29-32, wherein the second domain is N-terminal to the first domain.

41. A pharmaceutical composition capable of inducing or enhancing an antigen specific immune response, comprising the nucleic acid molecule according to any of claims 1-16 and a pharmaceutically acceptable carrier or excipient.

42. A pharmaceutical composition capable of inducing or enhancing an antigen specific immune response, comprising the expression vector of claim 17, and a pharmaceutically acceptable carrier or excipient.

43. A pharmaceutical composition capable of inducing or enhancing an antigen specific immune response, comprising the particle of claim 21, and a pharmaceutically acceptable carrier or excipient.

43. A pharmaceutical composition capable of inducing or enhancing an antigen specific immune response, comprising the cell of claim 25, and a pharmaceutically acceptable carrier or excipient.

44. A pharmaceutical composition capable of inducing or enhancing an antigen specific immune response, comprising the cell of particle of claim 26, and a pharmaceutically acceptable carrier or excipient.

45. A pharmaceutical composition capable of inducing or enhancing an antigen specific immune response, comprising the cell of claim 27, and a pharmaceutically acceptable carrier or excipient.

46. A pharmaceutical composition capable of inducing or enhancing an antigen specific immune response, comprising the cell of claim 28, and a pharmaceutically acceptable carrier or excipient.

47. A pharmaceutical composition capable of inducing or enhancing an antigen specific immune response, comprising the chimeric polypeptide of claim 29, and a pharmaceutically acceptable carrier or excipient.

48. A pharmaceutical composition capable of inducing or enhancing an antigen specific immune response, comprising the chimeric polypeptide of claim 33, and a pharmaceutically acceptable carrier or excipient.

49. A method of inducing or enhancing an antigen specific immune response comprising administering an effective amount of a composition comprising the nucleic acid of any of claims 1-16, thereby inducing or enhancing the antigen specific immune response.

50. A method of inducing or enhancing an antigen specific immune response comprising administering an effective amount of a composition comprising the expression vector of claim 17, thereby inducing or enhancing the antigen specific immune response.

51. A method of inducing or enhancing an antigen specific immune response comprising administering an effective amount of a composition comprising the particle of claim 21, thereby inducing or enhancing the antigen specific immune response.

52. A method of inducing or enhancing an antigen specific immune response comprising administering an effective amount of a composition comprising the cell of claim 25, thereby inducing or enhancing the antigen specific immune response.

53. A method of inducing or enhancing an antigen specific immune response comprising administering an effective amount of a composition comprising the chimeric polypeptide of claim 29, thereby inducing or enhancing the antigen specific immune response.

54. The method of claim 49, wherein the antigen specific immune response is mediated at least in part by $CD8^+$ cytotoxic T lymphocytes (CTL).

55. The method of claim 50, wherein the antigen specific immune response is mediated at least in part by $CD8^+$ CTL.

56. The method of claim 51, wherein the antigen specific immune response is mediated at least in part by $CD8^+$ CTL.

57. The method of claim 52, wherein the antigen specific immune response is mediated at least in part by $CD8^+$ CTL.

58. The method of claim 53, wherein the antigen specific immune response is mediated at least in part by $CD8^+$ CTL.

59. The method of claim 49, wherein the composition is administered *ex vivo*.

60. The method of claim 59, wherein the composition is administered *ex vivo* to APCs.

61. The method of claim 60, wherein the APCs are dendritic cells.

62. The method of claim 60 wherein the APCs are human APCs.

63. The method of claim 62, wherein the APCs are obtained from a live subject.

64. The method of claim 60, further comprising administering the *ex vivo*-treated APCs to a histocompatible subject.

65. The method of claim 49, wherein the composition is administered *in vivo*.

66. The method of claim 65, wherein the composition is administered to a human.

67. The method of claims 65, wherein the composition is administered intramuscularly, intradermally, or subcutaneously.

68. The method of claim 65 wherein the composition is administered intratumorally or peritumorally.

69. A method of increasing the number of $CD8^+$ CTLs specific for a selected desired antigen in a subject, comprising

administering an effective amount of a composition comprising the nucleic acid of any of claims 1-16, wherein the antigenic peptide comprises an epitope that binds to, and is presented on cell surfaces by, MHC class I proteins,

thereby increasing the number of said antigen-specific $CD8^+$ CTLs.

70. A method of increasing the number of $CD8^+$ CTLs specific for a selected desired antigen in a subject, comprising

administering an effective amount of a composition comprising the expression vector of claim 17, wherein the antigenic peptide comprises an epitope that binds to, and is presented on cell surfaces by, MHC class I proteins,

thereby increasing the number of antigen-specific $CD8^+$ CTLs.

71. A method of increasing the numbers of $CD8^+$ CTLs specific for a selected desired antigen in a subject comprising

administering an effective amount of a composition comprising the particle of claim 21, wherein the antigenic peptide comprises an epitope that binds to, and is presented on cell surfaces by, MHC class I proteins,

thereby increasing the numbers of antigen-specific $CD8^+$ CTLs.

72. A method of increasing the numbers of $CD8^+$ CTLs specific for a selected desired antigen in a subject comprising

administering an effective amount of a composition comprising the cell of claim 25, wherein the antigenic peptide comprises an epitope that binds to, and is presented on cell surfaces by, MHC class I proteins,

thereby increasing the numbers of antigen-specific $CD8^+$ CTLs.

73. A method of increasing the numbers of $CD8^+$ CTLs specific for a selected desired antigen in a subject comprising

administering an effective amount of a composition comprising the chimeric polypeptide of claim 29, wherein the antigenic peptide comprises an epitope that binds to, and is presented on cell surfaces by, MHC class I proteins,

thereby increasing the numbers of antigen-specific $CD8^+$ CTLs.

74. A method of inhibiting the growth of a tumor in a subject comprising administering an effective amount of a composition comprising the nucleic acid molecule of any of claims 1-16, thereby inhibiting growth of the tumor.

75. A method of inhibiting the growth of a tumor in a subject comprising administering an effective amount of a composition comprising the expression vector of claim 17, thereby inhibiting growth of the tumor.

76. A method of inhibiting the growth of a tumor in a subject comprising administering an effective amount of a composition comprising the particle of claim 21, thereby inhibiting growth of the tumor.

77. A method of inhibiting the growth of a tumor in a subject comprising administering an effective amount of a composition comprising the cell of claim 25, thereby inhibiting growth of the tumor.

78. A method of inhibiting the growth of a tumor in a subject comprising administering an effective amount of a composition comprising the chimeric polypeptide of claim 29, thereby inhibiting growth of the tumor.

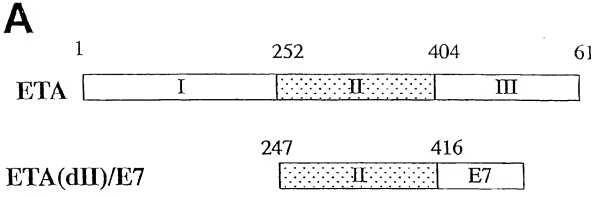
79. The method of claim 74, wherein the administering is intratumoral or peritumoral.

80. The method of claim 75, wherein the administering is intratumoral or peritumoral.

81. The method of claim 76, wherein the administering is intratumoral or peritumoral.

82. The method of claim 77, wherein the administering is intratumoral or peritumoral.

83. The method of claim 78, wherein the administering is intratumoral or peritumoral.



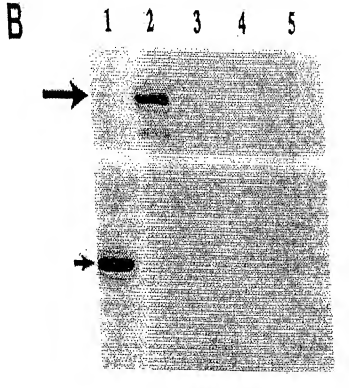


FIG. 1

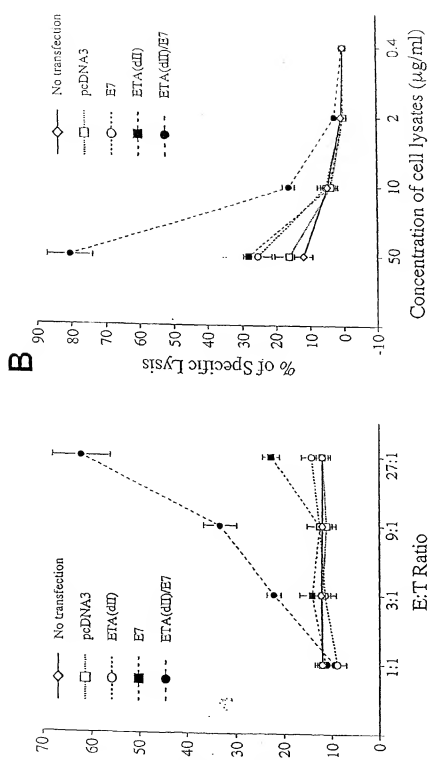

A
% of Specific Lysis
70
60
50
40
30
20
10
0
No transfection
pcDNA3
ETA(dII)
E7
ETA(dII)/E7
1:1
3:1
9:1
27:1
E:T Ratio
B
% of Specific Lysis
90
80
70
60
50
40
30
20
10
0
-10
No transfection
pcDNA3
E7
ETA(dII)
ETA(dII)/E7
50
10
2
0.4
Concentration of cell lysates (μg/ml)

FIG. 2

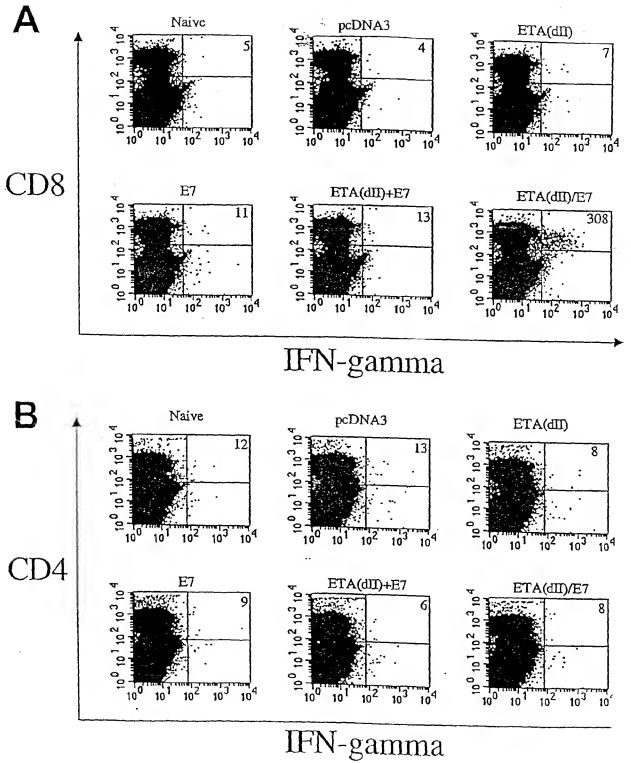

A
Naive
5
pcDNA3
4
ETA(dII)
7
CD8
E7
11
ETA(dII)+E7
13
ETA(dII)/E7
308
IFN-gamma
B
Naive
12
pcDNA3
13
ETA(dII)
8
CD4
E7
9
ETA(dII)+E7
6
ETA(dII)/E7
8
IFN-gamma

FIG. 3

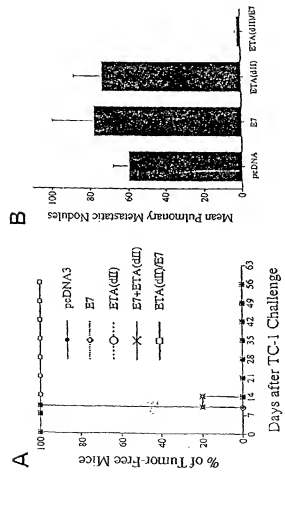

A
% of Tumor-Free Mice
Days after TC-1 Challenge
pcDNA3
E7
E7A(dII)
E7+E7A(dII)
E7A(dII)/E7
B
Mean Pulmonary Metastatic Nodules
pcDNA
E7
E7A(dII)
E7A(dII)/E7
C
% of Tumor-Free Mice
Days after TC-1 Challenge
Naive
No depletion
CD4 depletion
CD8 depletion
NK depletion

FIG. 4